Doctoral Student Research Committee (DSRC)

FIRST MEETING

Period: 07-09-2018 to 06-03-2019

Ph.D. Research Scholar: Vaishali Agrawal

Registration no.: 33/Ph.D./DPSRU/2018

Date of Enrolment: 26/02/2018

Date of Registration: 07/09/2018

Topic: Formulation Development of Various Agents to Improve its Efficiency as Internal Decorporation Agent

Supervisors

Prof. D.P. Pathak

Dr. A.K. Singh

DELHI INSTITUTE OF PHARMACEUTICAL SCIENCES AND RESEARCH

(Constituent college of Delhi Pharmaceutical Sciences and Research University)

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NEW DELHI-110017 INDIA

REPORT OF THE DOCTORAL STUDENT RESEARCH COMMITTEE (DSRC)

Name of the Research Scholar: Vaishali Agrawal

Registration no.: 33/Ph.D./DPSRU/2018

Conduct of the Research Scholar: Very Good

Overall Assessment of the progress and specific comments of the Supervisor:

She presented the research work meticulously All queries by the experts were answered by her.

13/5/2019
Prof. S.R. Wakode
Expert

Prof. S.P Aggarwal
Expert

Dr. Vibhav Garg
Expert

13.5.19
Prof. D.P. Pathak
(Supervisor)
Director, DIPSAR

Dr. A.K. Singh
(Supervisor)
Outstanding Scientist & DG, L
DRDO

Date: 13/5/19

Annexure A: Title and objective for approval
Annexure B: Progress report from date 07-09-2018 to 06-03-2019
Annexure C: Executive summary
Annexure D: Brief Review

Annexure A

Title: Formulation development of various agents to improve its efficiency as internal decorporation agent

Objectives

The objective of this study is to develop formulations of various agents to improve its efficiency as internal decorporation agent. The specific objectives aimed in the present work are as follows

- To develop and characterize a PB dosage form which should release the drug in duodenum for decorporation.
- To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vitro/ in-vivo* efficacy of developed formulation

Vaishali Agrawal
Name/Signature of Research Scholar

13.5.19
Supervisor:
Prof. D.P. Pathak
Director (DIPSAR)

13/5/19
Supervisor:
Dr. A.K. Singh
Outstanding Scientist & DG, LS
DRDO

Annexure B

PROGRESS REPORTFROM Date: 07-09-2018 to 06-03-2019

1. **Name of Research Scholar:** Vaishali Agrawal
2. **Correspondence Address**: Institute of Nuclear Medicine and Allied Sciences, Delhi-110054
3. **Contact No:** 9808851957
4. **Email:** agrawal.vaishali911990@gmail.com
5. **Registration No.:**33/PhD/DPSRU/2018
6. **Date of Registration:** 07-09-2018
7. **Semester Fee deposited**:** Rs 30385/- Dt.26-02-2018
8. **Approved Topic of research:**Formulation development of various agents to improve its efficiency as internal decorporation agent
9. **Nature of Fellowship, if any:** DRDO Fellowship (JRF)
10. **Details of leaves availed during the period under report, if any:**Copy attached
11. ***Research work done during the period:**Copy of detailed report attached

    a. PhD course work (Pharmaceutics)
    b. Literature review
    c. Selection of topic of research
    d. Selection of Drug
    e. Plan of research work

12. **Research papers:**

    a) Communicated research article entitled **"**Development and validation of LC-MS method for th estimation of N-Acetyl Tryptophan and its impurities under stress condition in*Chromatographia.*

13. *** Any other information:** Copy attached

- Awarded cash prize for Oral presentation on **"Development and validation of L MS method for the estimation of N-Acetyl- Tryptophan and its impurities under str conditions"**in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
- Poster presentation on **"Development and validation of LC-MS method for the estimat of N-Acetyl- Tryptophan and its impurities under stress conditions"**in 70th IPC held Amity University, Noida on 21-23 Dec 2018.

Vaishali Agrawal
(Signature of Research scholar)

Date: 13/5/19

13.5.19
Prof. D.P. Pathak
Signature of Supervisor

Dr. A.K. Singh
Signature of Sup

Annexure C

EXECUTIVE SUMMARY

A nuclear disaster during military, industrial, or medical practices leads to release of radionuclides which results in external and/or internal contamination with radioactive materials.Internal contamination occurs when radioactive materials have ingested, inhaled, or penetrated through skin or wound. After internalization, radionuclides are distributed to various tissues with patterns depending on the chemical and physical form of the radio nuclide. The pattern of injury is decided by the tissue distribution of a radionuclide and health status of exposed individuals.It is required to remove the radionuclide from GIT and systemic circulation by using surface active adsorbent or chelating agents. Decorporating agentis a broad term that includes all agents that can either prevent the incorporation of the radionuclides or decorporate the incorporated radionuclides. These agents are categorized as blocking agents, chelating agents, diluting agents and ion exchanger.The decorporating agents can be used for prevention of radionuclides absorption from g.i.t to systemic circulation, hastening the elimination of absorbed radionuclides from blood stream or decorporation of radionuclides from their target organs.

There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Potassium iodide for decontamination of iodine Calcium- and Zinc-diethylenetriaminepentaacetic acid (Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium). Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions, having high efficiency at high pH and site of action is duodenum (small intestine). Ca-DTPA is known for removal of actinides like plutonium, americium, curium and other bivalent ions. But no formulation is available in India. Thus these two decorporating agents were selected and their oral dosage form development needs to be carried out.

The available market formulation of PB i.e. "Radiogardase-Cs" is a conventional dosage form, having high dose. In one approach, modified oral dosage form of PB is designed with matrix tablet, having pH dependent release only in duodenumpreventing entero-hepatic circulation of cesium radionuclide and minimization of dose.Various parameters of matrix tablet will be evaluated like hardness, friability, drug content, *in-vitro* adsorption studies,

Pharmacoscintigraphy studies, *in-vivo* efficacy studies its comparison to Commercially available formulation "Radiogardase-Cs and i*n-vivo* safety studies.

Since, the only currently available chelating agents for decorporation of transuranic actinides are "Ca-DTPA and Zn-DTPA", available as i.v.DTPA has low oral bioavailability <3%.Therefore, in another approach, the oral formulation of Ca-DTPA is designed for decorporation for improving patient compliance, while dealing mass population during emergency. One of the possible formulation of **"SDEDDS"** which are the formulated mixtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are thermodynamically stable systems found to have great potential for BCS class III drugs, enhancing their oral bioavailability. Various parameters of SDEDDS formulation will be evaluated like viscosity analysis, emulsion droplet analysis, *in-vitro* drug release, *in-vitro* permeation studies,efficacy studies and its comparison to Commercially available formulation "Pentetate calcium trisodium injection 1000 mg , *In-vivo* absorption and pharmacokinetics studies.**In conclusion**the present study will explore the potential of matrix tablet of *Prussian*blue for the removal of cesium radionuclides with its target release in duodenum and reduction of dose. Secondly SDEDDS and other approaches for enhancing oral bioavailability will be explored for Ca-DTPA. Both formulations will be optimized in order to enhance better decorporation efficiency for target radionuclides.

Annexure D

BRIEF REVIEW

There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)**for decontamination of cesium, Calcium- and Zinc-diethylenetriaminepentaacetic acid **(Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer)** for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium).

Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions. `It is a crystal lattice that exchanges potassium for cesium at the surface of crystal **(Thompson & Callen, 2004).** It has been used both in animals **(R. Barroso-Moguel et al., 1994)**and clinically in humans **(Pau, 2000).** Radioactive cesium (^{137}Cs) is the most harmful naturally occurring radionuclide, with a long half-life (30.17 years) and high water solubility and mobility, which readily enters the animal and human food chains through the consumption of contaminated water, plants, meat, fish, and milk **(Ansoborlo & Wayne, 2015).** Moreover, Cs in animals and humans is processed pharmacokinetically in the same way as sodium (Na) and potassium (K) owing to its chemical analogy with those elements **(Chen et al., 2013)**.The ability and ion–exchange property of Prussian blue plays an important role in trapping heavy metal ionsand enhancing their faecal excretion **(Reddy et al., 1996, Schwudke et al., 2000, Zakharchuk, 1999).** After the 1987 contamination incident in Goiania, Brazil, Prussian blue was used successfully, reducing whole-body committed radiation doses by up to a factor of **two (Melo et al., 1996; Farina &Brandiio-Mello, 1991).**The efficacy of PB has been reported at high pH.

The only currently available chelating agents for decorporation of transuranic actinides are **"Ca-DTPA and Zn-DTPA".** They forms stable chelates with metal ions by exchanging calcium and zinc respectively for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. Due to its charge and hydrophilicity, DTPA shows poor oral bioavailability (approximately 3%) **(Volf, 1984).**

Annexure E

List of Publications

- Vaishali Agrawal, Ruchi Baghel, A.KSingh, D.P Pathak and Nidhi Sandal*, "Development and validation of LC-MS method for the estimation of N-Acetyl Tryptophan and its impurities under stress conditions", Chromatographia, 2019 (Communicated).
- Awarded cash prize for Oral presentation on "Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
- Poster presentation on "Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.

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Introduction:

Accidents involving nuclear reactors, industrial sources, or medical sources leads to internal radioactive contamination **(USFDA, 2006).** The different modes of internalization of radionuclides may be inhalation, ingestion, injection or absorption through intact or abraded skin. After internalization, radionuclides are distributed to various tissues with patterns depending on the chemical and physical form of the radio nuclide. The pattern of injury is decided by the tissue distribution of a radionuclide and health status of exposed individuals **(Cassatt et al., 2008)** The retention time of radioactive contaminants in the body decide the extent of significant health risk pose to the body. Thus the removal of such radioactive contaminants is the only method to reduce such health risks **(USFDA, 2006)**.

The aim of internal decorporation is to reduce absorption and enhance excretion of radioactive contaminants. Decorporation agents refers to drug products that increase the rate of elimination or excretion of absorbed, inhaled, or ingested radioactive contaminants **(USFDA, 2006)**. Decorporating agents are a broad term that may either prevent incorporation of radionuclide or decorporate the incorporated radionuclide. These agents may be categorized as blocking agents, chelating agents, diluting agents, ion exchanger. Decorporation agents can be used at three levels:

1. Prevention of radionuclides absorption from git to systemic circulation.
2. Hastening the elimination of absorbed radionuclides from blood stream.
3. Decorporation of radionuclides from target organs.

Removal of internalized radionuclide is a vast problem due to: Large number of radionuclides, their different chemical form and solubility, physical decay, multiple route of entry, bio kinetics and organ specificity. Complete removal of internalized radionuclide from body is rather impossible, thus we intend to develop a ready to use clinically validated decorporation agents formulated in desired dosage form which can be used by military people for decorporation of internalized radionuclides. The radioisotopes, their chemical form and their amount cannot be predicted, therefore decorporation agents that can provide maximum protection were used. There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Potassium iodide for decontamination of iodine Calcium- and Zinc-diethylenetriaminepentaacetic acid (Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals,

Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium). Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions and Ca-DTPA for removal of actinides like plutonium, americium, curium and other bivalent ions. Thus these two decorporating agents were selected and their oral dosage form development needs to be carried out.

Prussian blue (Ferric ferrocyanide) is a dye used for internal decorporation of Cs-137 and ^{201}Tl. It is a crystal lattice that exchanges potassium for cesium at the surface of crystal It has been used both in animals **(R. Barroso-Moguel et al., 1994)** and clinically in humans **(Pau, 2000).** Radioactive cesium (^{137}Cs) is the most harmful naturally occurring radionuclide, with a long half-life (30.17 years) and high water solubility and mobility, which readily enters the animal and human food chains through the consumption of contaminated water, plants, meat, fish, and milk **(Ansoborlo & Wayne, 2015).** Moreover, Cs in animals and humans is processed pharmacokinetically in the same way as sodium (Na) and potassium (K) owing to its chemical analogy with those elements **(Chen et al., 2013)**. Approximately 10% of Cs is eliminated rapidly with a biological half-life of 2 days, 90% is eliminated gradually with a biological half-life of 110 days, and less than 1% remains with a longer biological half-life of approximately 500 days15. These type of insoluble contaminants target the gastrointestinal (GI) tract traveling the entire length of the tract unabsorbed and excrete via hepatobiliary clearance. Some nonabsorbed binding resins find utility in inhibiting the uptake of radioactive contaminants in the gut. E.g. Prussian blue after oral intake, not absorbed by the GI tract, binds cesium ions in the gut enhancing its fecal excretion. After the 1987 contamination incident in Goiania, Brazil, Prussian blue was used successfully, reducing whole-body committed radiation doses by up to a factor of **two (Melo et al., 1996; Farina & Brandiio-Mello, 1991) .**

The efficacy of PB has been reported at high pH. Therefore the purpose of the study is to target the drug at duodenum of small intestine and also avoid overdosing of PB.

Ca-DTPA and Zn-DTPA forms stable chelates with metal ions by exchanging calcium and zinc respectively for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. The chelating capacity of Ca-DTPA is greatest immediately and approximately 24 hours after internal contamination when the radio contaminants are still circulating and readily available for chelation. After the first dose of Ca-DTPA, maintenance dose of CA-DTPA or Zn-DTPA resulted in similar rates of elimination but Ca-DTPA was found to be associated with greater toxicity, including more depletion of trace

metals, higher rate of mortality, presence of kidney and liver vacuolization, and small bowel hemorrhagic lesions. The recommended initial dose of Ca-DTPA in adults and adolescents is 1 g administered intravenously. In patients younger than 12 years of age, a single initial dose of 14 mg/kg (not to exceed 1 g) should be administered intravenously. These salts are not found to be effective for decorporation of uranium, neptunium or radioactive iodine **(Cada, 2005).**

Both Ca-DTPA and Zn-DTPA are poorly absorbed following oral administration. DTPA is not very effective when administered orally possibly due to the low absorption of DTPA through the intestines. It belongs to BCS class III category. It has very low oral bioavailability **(approx. 3%) (Abergel, 2016; Durbin, 2008; Taylor, Hodgson, & Stradling, 2007).**

Therefore the purpose of the study is to develop the oral formulation of Ca-DTPA in order to improve patient compliance and ease of administration. One such design may be the formulation of "self-double emulsifying drug delivery system (SDEDDS), which are the formulated mixtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are thermodynamically stable systems found to have great potential for BCS class III drugs, enhancing their oral bioavailability **(Qi et al., 2011).**

Literature Review

1. Internal decorporation

Once radioactive materials cross cell membranes, they are said to be incorporated. It is time dependent phenomenon, related to both physical and chemical nature of contaminant. The time of incorporation can be rapid, or minutes or days to months, but decorporation should be urgent in order to reduce health effects **(E.A. Waller, 2002).** The aim of internal decorporation is to reduce absorption and enhance excretion of radioactive contaminants. Decorporation agents refers to drug products that increase the rate of elimination or excretion of absorbed, inhaled, or ingested radioactive contaminants **(USFDA, 2006).** Decorporating agents is a broad term that may either prevent incorporation of radionuclide or decorporate the incorporated radionuclide. These agents may be categorized as blocking agents, chelating agents, diluting agents, ion exchanger. Decorporation agents can be used at three levels:

a) Prevention of radionuclides absorption from git to systemic circulation.
b) Hastening the elimination of absorbed radionuclides from blood stream.
c) Decorporation of radionuclides from target organs.

The main purpose of radionuclide decorporation is to minimize the radiation dose received by exposed individuals. There are certain metal radionuclides which when deposited in bone, liver or kidneys shows very long biological half-lives, thus rapid initiation of chelation treatment is necessary so that uptake from skin, lungs, git, will get reduced and excretion will get promoted before tissue deposition **(Andersen, 2016).**

Oral Prussian blue is the choice for Cesium decorporation, used extensively in highly contaminated patients from Goiania incident and resulted in whole body radiation by factor of 2 **(Brandao-mello, 1991; lipsztein et al., 1991; Oliveira et al., 1991; Farina, & Brandiio-Mello, 1991).** PB will bind radionuclides in the git, making it unavailable for absorption **(Bhattacharya et al., 1992).**

For Americium and Plutonium, Chelating agents like DTPA is the standard treatment available **(Andersen, 2016).**

Presence of radioactive contaminants in the GIT, can be eliminated by speeding up intestinal transit, which favor excretion in stool rather than absorption e.g. Simple laxatives.

Flooding the git with stable counterparts of the radioactive material will compete with the radioactive material for absorption.

Once radionuclide enters the blood, it can be block to uptake in the target organ by using non-radioactive potassium iodide to block radio iodide incorporation in to thyroid hormones and subsequent storage in the thyroid gland **(Holland, 1963).**

2. Radioactive contaminants

2.1 Cesium radionuclide

Glenn T. Seaborg and Margaret Melhase discovered Cesium-137, a radioactive isotope of cesium was discovered in 1941. Cesium-137 (^{137}Cs) make severe hazards for several generations with has a physical half-life of 30 years with β-energy peak at 174 Kev **(Jacques M. Verzijl, 1993; Rosoff et al., 1963).**

The most notable radioactive nuclide releases in to the environment is "Caesium-134 and caesium-137. These were found to be released in all nuclear weapon tests and some nuclear accidents, most notably the Chernobyl disaster and the Fukushima Daiichi disaster. Together with caesium-134, iodine-131, and strontium-90, Caesium-137 was found to be the major isotopes distributed by the reactor explosion that constitute the greatest risk to health. **(Wikipedia).**

After FNPP disaster, multitude of different radioactive materials are released into the environment. The strongest effect was found to be of radioactive strontium and cesium after several years. But radioactive cesium was regarded as the greatest threat to human exposure in Japan because the amount of radioactive strontium emitted during the FNPP disaster was comparatively low **(Hayano et al., 2013).**

(Leggett, 1986) found the route of distribution of cesium in body. It was found to follow potassium closely and its higher concentration was found in liver, skeletal muscles and red blood cells. Typical symptoms related to gastrointestinal effects include severe nausea, vomiting and diarrhea and systemic involvement leads to bone marrow suppression, hemorrhage and death **(Jacques M. Verzijl, 1993; Oliveira et al., 1991; Farina & Brandiio-Mello, 1991; Roberts, 1987).**

A radioactive element has a constant rate of disintegration that is reflected by its physical half-life. The rate of element elimination from the body is reflected by its biologic half-life. The combined rate of radiation disintegration and rate of element elimination is reflected by the effective half-life. Cesium follows the movement of potassium and is excreted into the intestine,

reabsorbed from the gut into the blood, then to the bile, where it is excreted again into the gut (enterohepatic circulation) **(Radiogardase, FDA).**

Cesium is well absorbed orally, primarily excreted through kidneys (with 10% fecal excretion) following first order elimination **(Rosoff et al., 1963; Ioannides et al., 1996).**The physiologic half-life of cesium excretion in humans is 50-150 days. Approximately 10% cesium is eliminated with biological half-life (2 days); 90% (110 days); less than 1% (500 days) **(Leggett, 1986; Taylor Mary P., 1962)**. Because of cesium's long physical half-life, the rate of radiation elimination is similar to the rate of element elimination from the body **(Radiogardase, FDA).**

(Lipsztein et al., 1991) suggest that with increasing weight, cesium elimination half-life increases.

2.2 Actinide series radionuclide

All isotopes of the 15 elements of Actinide series are (Atomic numbers 89 through 103) are radioactive and have the potential to be harmful. The most likely encountered ones are Uranium (U), Neptunium (Np), Americium (Am), Plutonium (Pu), Curium (Cm). Internalized actinides have the potential to induce both radiological and chemical toxicities, leading to serious health effects. They possess no known essential role in the normal biochemical reactions occurring in living organisms and are a particular hazard due to their ionizing radiation properties. Independent of contamination route (ingestion, inhalation or wound), actinides are absorbed then transported by blood prior to deposition in the target organs (Bone, liver and kidney), in which they are stored and partially excreted through urine and faeces. All actinides are radioactive and most of their isotopes decay by alpha particle emission **(Abergel, 2017).**

At Hanford site nuclear waste management facility, explosion in ^{241} Americium ion exchange column took place which causes extensive contamination of male worker **(Robinson et al., 1976).**

3. Decorporation agents

3.1 For Cs^+/Tl^+ monovalent ions decorporation

(Nilchi et al., 2003) studied many compounds which are highly selective ion exchange for cesium.

(Taj et al., 2011) found that Prussian blue is best for the removal of cesium.

(Gall et al., 2006) confirmed that PB increases excretion of cesium five folds and reduced the Cs levels in main organs by comparing the efficacy of apple-pectin and Prussian blue**.**

(Fekry et al., 2003) studied the role of Prussian Blue in reducing the physiological effects of Cs internal contamination and found that PB administration before or at the time of irradiation could eliminate the decreased or increased effects of [Cs]-gamma irradiation on: RBCs, Hb, total proteins, globulins, creatinine, urea, urea nitrogen, SAT, T_3 , T_4 .

(Brenot & Rinaldi, 1967) found that Cesium readily binds to Prussian blue (PB). Administration of single dose of radiocesium and the concominant oral application of PB results in reduction of cesium uptake from GIT. In piglets it was found to be more than 97%. The action of reduction of Cs-137 body burden depends on the dose and post exposure timing of dose.

After treatment with PB, most of the incorporated radio cesium is eliminated through faeces rather than urine resulting in decreased biological half-life by 50% (in rats from 11 days to 6 days; in dogs from 11 to 6.5 days). These animal results parallel human kinetic data noted in the radiological accident in Goiania **(Richmond, 1983).**

(Oliveira et al., 1991) reported the efficacy of PB in Goiania patients. On increasing the dose of PB, resulted in increased activity in fecal samples. The dose of PB administered inverse the ratio of feces to urine from 1:4 to 4:1.

(Lipsztein et al., 1991a) concluded the first order linear kinetic pattern of Cs-elimination both without and with dosing of PB. After studying various factors such as height, weight and age, he concluded that the elimination half-life to be mostly affected by weight.

(Stather, 1972) showed PB to bind Cs-137 in lumen of mammalian gut forming a stable complex and thus increasing the fecal loss of Cs-137.

PB was used along with sodium perchlorate and sodium alginate for the simultaneous decrease of cesium, iodine and strontium retention respectively. There was found no interference between additives. Alginates, sodium perchlorate and PB produced appreciable reduction in the treatment of oral doses of radioactive ^{85}Sr, ^{131}I and ^{137}Cs respectively **(Kostial et al., 1980).**

(Ioannides et al., 1986) investigated the effectiveness of PB in reducing Cs-137 contamination in ovine tissues and found that most of Cs-137 binds to PB in the gut and excreted in feces.

(Melo et al., 1996) studies efficacy of PB in beagles contaminated with Cs-137 and found that the half –life decreased from 29 d to 13 d in treated dogs.

3.2 For Actinides and other bivalent ions decorporation

The only currently available chelating agents for decorporation of transuranic actinides are "Ca-DTPA and Zn-DTPA". These are available as 0.5g vials by Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium).

During the Hanford incident (1976), the intake of 241 Am by skin absorption and inhalation in nuclear workers was in excess of 1 mCi, which was prevented (up to 99%) from entering the blood stream and being deposited in the internal organs by intensive DTPA therapy **(Robinson et al., 1976).**

Ca-DTPA forms stable chelates with metal ions by exchanging calcium for a metal of greater binding capacity. Likewise, Zn-DTPA forms stable chelates with metal ions by exchanging zinc for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. When given within hour of contamination, Ca-DTPA resulted in about a 10-fold higher rate of elimination of plutonium in the urine than Zn-DTPA.The chelating capacity of Ca-DTPA is greatest immediately and approximately 24 hours after internal contamination when the radio contaminants are still circulating and readily available for chelation. After the first dose of Ca-DTPA, maintenance dose of CA-DTPA or Zn-DTPA resulted in similar rates of elimination but Ca-DTPA was found to be associated with greater toxicity, including more depletion of trace metals, higher rate of mortality, presence of kidney and liver vacuolization, and small bowel hemorrhagic lesions **(Abergel, 2006).**

(Volf, 1986) compared the efficacy of 3, 4, 3 LICAM with that of Ca-DTPA and deferoxamine and found Ca-DTPA superior to both in decorporation of ^{241}Am when administered early after incorporation and used at its clinical doses (30micromol/ kg B.W.)

(Cohen et al., 1979) found that chelation therapy with Ca-DTPA for 8 years effectively reduce body burden of ^{241}Am ion in the child (4yr old) rather than father (50yr old). They got contaminated via inhalation with Americium in home.

These salts are not found to be effective for decorporation of uranium, neptunium or radioactive iodine **(Cada, 2005).**

4. Drug profile

4.1 Prussian blue

4.1.1 Chemistry:

Prussian blue is a dark blue synthetic pigment produced by oxidation of ferrous ferrocyanide salt. It contains Ferric ferrocyanide (ferric hexacyanoferrate II) in a cubic lattice structure. Its chemical formula is Fe_4 [Fe (CN) $_6]_3$(Mwt: 589.3 Da).It was first synthesized in 1704 by a Berlin color maker named. The crystal structure of PB is a cubic lattice with the Fe (II) and Fe (III) atoms occupying corners of the cube and cyanide groups positioned on the sides. Its crystalline structure was established on the basis of its powder diffraction pattern **(Keggin & Miles, 1936).**

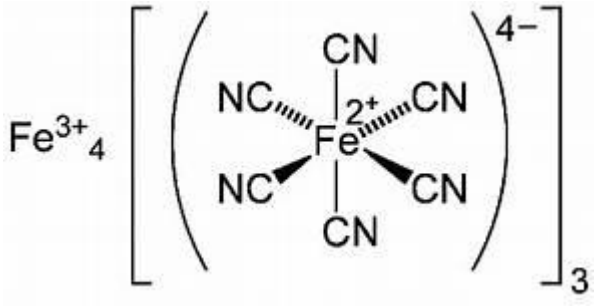


Fig. 1 Structure of Unit cell of Prussian Blue

PB is a mixed valence species with basic cubic structure in which high spin iron (III) ligated octahedrally by six nitrogen atoms and low spin iron (II) ligated by six carbon atoms of cyanide in a face centered cubic lattice so effectively that intense charge transfer occurs **(Crumbliss et al., 1984; Karyakin, 2001).** This is the reason for deep blue color. Channels run through the solid and connect holes so as to give PB a porous structure very similar to that of zeolites, the holes of PB are filled partially with hydrated cations. Prussian blue is classified as both soluble and insoluble, depending on the absence or presence of a monovalent ion in the lattice. Thus, Fe4 [Fe (CN) 6]3 is known as insoluble whereas KFe_3 [Fe $(CN)_6$]3 is soluble Prussian blue **(Buser et al., 1977).**

Prussian blue has the ability to form a wide range of simple and very complicated solutions with other metal hexacyanoferrate like nickel, copper and cadmium. This ability and its ion – exchange properties plays an important role in trapping other heavy metal ions (Schwudke , Stober and Scholz, 2000) (**Reddy et al., 1996, Schwudke et al., 2000, Zakharchuk, 1999).**

4.1.2 Characterization

PB is insoluble in water, organic or inorganic solvents, sparingly soluble in 1M oxalic acid **(Neil Merck, 2001)**.It is characterized by IR, TEM and RD studies.

Since PB is insoluble in water, organic and inorganic solvents, it can be determined spectrophotometrically by following two-step dissolution process. It can be seen in Fig. 2.

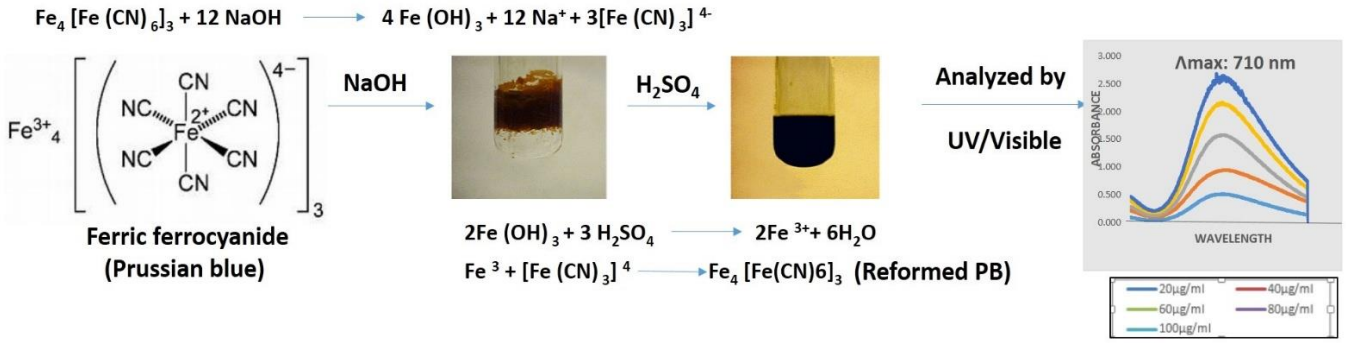


Fig. 2 Acid-Base treatment of insoluble PB for analytical determination

In I.R spectrum. The absorption band at around 2085 cm^{-1} is a CN stretching vibration typical of solid PB **(Furniss, 1989).** IR spectrum of insoluble PB shows an intense peak at 2087 cm^{-1} **(Scholz, et al., 2001).**

4.1.3 Mechanism of action and pharmacokinetics

Prussian blue insoluble, ferric (III) hexacyanoferrate (II), after oral ingestion is not absorbed through the intact gastrointestinal wall. Its clearance from the body depends on the gastrointestinal tract transit time. Prussian blue insoluble acts by ion-exchange, adsorption, and mechanical trapping within the crystal structure and has a very high affinity for radioactive and non-radioactive cesium and thallium. Prussian blue insoluble binds cesium and thallium isotopes in the gastrointestinal tract after these isotopes are ingested or excreted in the bile by the liver thereby reducing gastrointestinal reabsorption (enterohepatic circulation). In studies of rats, pigs, and dogs that were internally contaminated with cesium and thallium, the presence of the insoluble complexes in the gastrointestinal lumen, changed the primary elimination route from the kidney to the feces and increased the rate of elimination of these two contaminants. The rate of cesium and thallium elimination was proportional to the duration and dose of Prussian blue insoluble.

Without Prussian blue insoluble treatment, ~80% of cesium is excreted through the kidneys and ~20% in the feces. Based on the mechanisms of action, Prussian blue insoluble may bind other elements (e.g., potassium), and cause electrolyte or other nutritional imbalances.

4.1.4 Dose and administration

Adults and Adolescents The recommended dose of Prussian blue insoluble is 3 grams orally three times a day.

Pediatrics (2 – 12 years) The recommended dose of Prussian blue insoluble is 1 gram orally three times a day.

4.1.5 Adverse effects

PB is relatively nontoxic when given orally **(Pearce et al., 1994).** Mild cases of hypokalemia have been reported because potassium is exchanged for cesium at the surface of the crystal lattice. Thus potassium replacement has been done for normal level **(Farina & Brandiio-Mello, 1991).** She also found 20% frequency of constipation in 46 patients who received PB in Goiania tragedy for radiocesium decorporation. So light laxatives should be recommended along with PB so that time duration of contaminated feces with lumen decreased.

Constipation can be seen in PB therapy patients so administering 15% mannitol along with PB advised for adequate fecal movement **(Thompson, 1981).**

4.2. Ca-DTPA

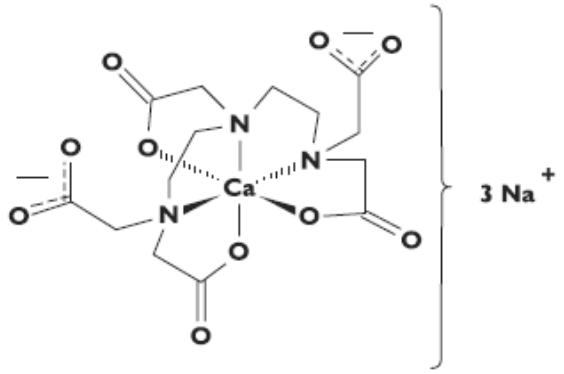
4.2.1 Chemistry (Rx list and Drug bank; Cada et al., 2005; Package insert, FDA, 2004; CDC, 2006)

Synonym: Trisodium calcium diethylenetriaminepentate (Schwudke & Stoßer, 2000)-acetate; Pentetate calcium trisodium

Molecular formula: $Na_3CaC_{14}H_{18}N_3O_{10}$

Molecular weight: 497.4 Da

Structural formula:



Mechanism of action: Ca-DTPA forms stable chelates with metal ions by exchanging calcium for a metal of greater binding capacity. The radioactive chelates are then excreted by glomerular filtration into the urine. In animal studies, Ca-DTPA forms less stable chelates with uranium and neptunium in vivo resulting in the deposition of these elements in tissues including the bone. Ca-DTPA treatments are not expected to be effective for uranium and neptunium. Radioactive iodine is not bound by DTPA.

Water Solubility: 11mg/mL

LogP: 0.22

Dose: A single 1.0-gram initial dose of Ca-DTPA (Pentetate calcium trisodium inj) administered intravenously.

Available Dosage form in market: Ca-DTPA (Pentetate calcium trisodium inj) is supplied as a sterile solution in 5 mL single-use clear glass ampoules at a concentration of 200 mg/mL for intravenous use. Each ampoule contains the equivalent of 1000 mg of Pentetate calcium trisodium.

Pharmacokinetics: Ca-DTPA (Pentetate calcium trisodium inj) is poorly absorbed in the GI tract (approximately 5%). Following intravenous administration, it is rapidly distributed throughout the extracellular fluid space. No significant penetration in to erythrocytes or other cells or no accumulation in specific organs has been observed. Little or no binding of it by renal parenchyma. Ca-DTPA is cleared from the plasma in the first few hours after dosing through urinary excretion by glomerular filtration. The main adverse metabolic effects of Ca-DTPA is that it binds endogenous metals of the body (Zn, Mg and Mn).

4.2.2 Characterization

(Yu et al., 2018) develop a robust LC-MS/MS method to quantify Zn-DTPA, a zinc chelate in human plasma and urine. It has been successfully applied to quantification of Zn-DTPA in plasma and urine samples after intravenous infusion administration of Zn-DTPA to a male healthy volunteer and results confirm that the kidney rapidly and completely eliminates Zn-DTPA in human.

(Wang & Tomasella, 2016) studied Ion-pairing HPLC methods to determine EDTA and DTPA in small molecule and biologic pharmaceutical formulations. Because EDTA and DTPA do not contain chromophores, transition metal ions like Cu^{2+}, Fe^{3+} should be added respectively to enhance metal detection. The analytical method development was designed based on the chromatographic properties of the analytes, the nature of the sample matrix and the intended purpose of the method.

(Laine & Matilainen, 2005) studied simultaneous determination of DTPA, EDTA, and NTA by UV–visible spectrometry and HPLC. This study shows that spectrophotometry and high-performance liquid chromatography can be used for simultaneous determination of DTPA, EDTA, and NTA in dishwashing detergents, and that the total amount of complexing agents can be determined by potentiometric titration.

(Kamboj et al., 2011) studied a simple, sensitive, economical and reproducible UV-Spectrophotometric method for the estimation of DTPA in topical gel formulations. The method is based on NaFeDTPA complex formation by the reaction of DTPA with ferric chloride in 0.1N HCl. The procedure is accurate, precise and reproducible (relative standard deviation < 1 %), while being simple, cost effective and less time consuming, which proves the suitability of the proposed method for the estimation of DTPA in topical gel formulations.

5 Dosage form development

5.1 Prussian blue

Decorporating agents belong to exclusive class of drugs, not dealt by any pharmaceutical scientists. These are given either as powder in water or parenterally by i.v or i.m injections. PB is most extensively studied and used decorporating agents, only available as Radiogardase-Cs capsules. It is a type of conventional release dosage form, with recommended dose (3-10g) per day. This is very high dose due to which poor patient compliance and constipation adverse effects seems to be reported. Thus high dose needs to be reduced.

(Arun & Joseyphus, 2014) developed Prussian blue modified Fe3O4 nanoparticles for Cs detoxification. The Cs removal efficiency reached maximum within 120 min confirmed by Cs ion adsorption studies. Factors affecting capture efficiency of the prepared nanoparticles was studied by varying the flow channel diameter, applied magnetic field, and the fluid flow velocity. The study suggests that PB-Fe3O4 nanoparticles could be used for the detoxification of Cs where the flow velocity is in the range of few tens of cm/s.

(Uemura & Kitagawa, 2003) developed Prussian Blue Nanoparticles protected by Poly (vinylpyrrolidone). They successfully developed the PVP-protected PB nanoparticles by mixing $Fe^{2+,}$ Fe $(CN)_6^{3-}$ and PVP in water. The dispersed nanoparticle formations in the polymer matrix offered PB not only the enhanced solubilities in organic solvents but also a film-forming property when the colloidal dispersions of the composites were evaporated.

(Hoffman et al., 1999) reported 72.2mg/g maximum adsorption efficacy of PB by in-vitro adsorption studies of PB and Thallium, which states that maximum $5.55*10^{10}$ mCi of Tl can be decorporated by 1g of PB. This figure of radioactivity is the extreme number beyond which radioactivity was not even released during Chernobyl and Goiania incidents.

Therefore intake of such high dose should be reduced. PB found to act on intestinal lumen **(Groot et al., 1985; Farina & Brandiio-Mello, 1991)** and better efficacy is reported at higher pH. Thus present attempt is to formulate matrix tablet of PB which release drug only in small intestine (duodenum) and present better efficacy at lower dose. For designing oral dosage form for PB, variety of polymers have been reviewed.

Since we all know, Oral route is the most preferable route with better patient compliance, ease of administration, and flexibility in formulation. pH sensitive and matrix polymers need to be considered. A matrix system is a kind of simple system which resist drug dumping and attain end target drug release. They are easy to formulate. Variety of polymers are already well documented like Sodium alginate, HPMC phthalate, Cellulose acetate phthalate, Eudragit R, Eudragit RS etc.

5.2 Polymer review

5.2.1 Sodium alginate (Na Alg)

It is a water soluble salt of alginic acid, a natural polysaccharide extracted from marine brown algae. It contains 2 uronic acidsβ-D-mannuronic acid (M) and α-L-glucoronic acid (G). It contains either homopolymeric blocks (MM or GG) or heteropolymeric blocks (MG) **(Aslani & Kennedy, 1996; Yotsuyanagi et al., 1991)** Alginic acid is insoluble in aqueous media, Ad pH is

raised over 3, and partly soluble salt is formed. As pH is raised around 4, its soluble salt is formed. Sodium, magnesium and ammonium salts are examples of water soluble alginate salts. Neutralization with calcium, barium and other bivalent metal ions produce insoluble alginate salts. In gastric acid, it gets hydrated and forms porous, insoluble alginic acid while in higher pH the alginic acid gets converted in to soluble viscous layer. It means the gel shrinks at acidic pH and erodes at alkaline pH. Thus it can be used effectively to deliver drugs to intestine.

(Rubio, 2015; Tomida et al., 1993) showed that sodium alginate can be used for matrix entrapment of drugs. It forms hydrophilic gels by interaction with bivalent metal ions.

Alginate gel beads can be formed by dropping solutions of sodium alginate in to solutions of calcium chloride **(Badwan et al., 1985; Kim & Lee , 1992).**

Lin et al., 2004 prepared microencapsulated beads of alginate blended with chitosan by dropping aqueous alginate-chitosan solution in to calcium solution. These beads were evaluated for pH-sensitive delivery of model drug (BSA, Bovine serum albumin).

Li et al., 2015 reported chitosan –sodium alginate based matrix tablets for extended release of highly water soluble drugs like trimetazidine hydrochloride (TH).

Moreover, alginates have been reported to bind strontium **(Hesp & Ramsbottom, 1965).**

Thus formulation development using sodium alginate was given priority due to its added advantage of strontium binding and pH dependent erosion.

5.3 Ca-DTPA

5.3.1 Formulation development

The main factors responsible for DTPA's permeability limited absorption and resulting poor oral bioavailability (approximately 3%) are Charge and hydrophilicity **(Volf, 1984).**

Several attempts have been made to increase the lipophilicity of DTPA through esterification of carboxylic acid moieties **(Durbin, 2008).**

"Puchel", an early lipophilic derivative of DTPA which incorporated two undecanoic acid chains in order to enhance intracellular penetration was designed, no actinide-removal efficacy enhancement or toxicity reduction was observed *in vivo*, however, in comparison to Ca-DTPA **(Peter & Volf, 1981).**

Added long alkane chains to triethylenetetramine hexaacetic acid (TTHA), a nonadentate analog of DTPA to achieve oral bioavailability enhancements .Among that series of C*n*TT compounds (*n* varying from 8 to 22 carbons), the C16TT and C22TT chelators were found most effective at reducing 241Am and 239Pu content in the tissues of contaminated rats, but were not more effective or less toxic than Ca-DTPA **(Miller et al., 2005).**

More recently, while focusing on bioavailability enhancement, Jay and coworkers prepared and evaluated a series of DTPA esters for their physico- chemical properties and permeability characteristics **(Sueda et al., 2015; J.E, 2014; Sueda et al., 2012).**

Initially emerged candidates for further development are "penta- ethyl and di-ethyl esters of DTPA, referred to as C2E5 and C2E2" respectively. C2E2 is still under investigation as a promising oral chelator for transuranic elements **(J.E, 2014).**

To enhance oral delivery **(Shankar et al., 2014)** developed Zn-DTPA tablets, an oral solid dosage form containing permeation enhancers. Limited efficacy and safety studies are available for these tablets, however recent publications reported that daily oral administration at a 1325 mg−1 kg−1 day−1 level for 7 days was well tolerated in Beagle dogs. In addition, decorporation efficacy in rats injected with 241Am and treated once with oral tablets (575 μmol kg−1) or intravenous DTPA (30 μmol kg−1) was comparable.

Patent **WO 2007145682 A1** by **G. Natarajan Shankar, H. Jaber Parish (2007),** Oral DTPA for radionuclide chelation was patented. A composition for oral radionuclide chelation therapy comprises a DTPA chelate selected from Zn-DTPA and Ca-DTPA and a permeation enhancer that preferentially increases jejunal uptake of the DTPA chelate. The composition has a DTPA chelate bioavailability of at least 10% of the chelate when orally administered to a mammal.

Nano therapeutics Inc. developed nano-particulate aggregates of DTPA and zinc acetate by encapsulating with-in entero-coated capsules, providing the most advanced oral delivery product for DTPA, NanoDTPA ®. Under this form, DTPA displayed significantly improved bioavailability in dogs and promoted as much 241Am removal as intravenous DTPA **(Reddy et al., 2012; Wilson et al., 2015)**.

So, one of the major limitations of DTPA is its need to be administered by intravenous injection or inhalation. Such treatments require skilled medical personnel for administration, and in emergency situations involving mass-casualties, the product is not conducive to rapid distribution and self-administration. DTPA is proven to be most effective when the treatment

commences soon after contamination; delaying the treatment can significantly reduce the overall efficacy, which can only be compensated partially by repeated treatments over time. An orally administered dosage form can alleviate these limitations. However, the oral absorption of DTPA is hampered by it hydrophilic nature, resulting in permeability-limited absorption. Thus, the poor oral bioavailability (3%) of DTPA makes it unsuitable as a product for oral administration. **(Sueda et al., 2012).** Therefore, one of the possible way to formulate oral dosage form of Ca-DTPA is "Self double emulsifying drug delivery system". These w/o/w double emulsions show great potential for enhancing the oral bioavailability of low-permeability compounds. The emulsion can protect hydrophilic bioactive substances from degradation and can penetrate the small intestinal epithelium (Qi et al., 2011). Water-in-oil-in-water (w/o/w) double emulsions are complex systems consisting of aqueous droplets dispersed within larger oil droplets, which are themselves dispersed in an aqueous continuous phase. The internal aqueous droplets encapsulated by the oil membrane can be seen as a storage chamber for hydrophilic drugs. This structure could protect the drug dissolved in the internal aqueous phase. But due to certain limitations like thermodynamic instability with strong tendency towards coalescence, flocculation, creaming, instability against heat, organic solvents and pH changes, a novel formulation design was developed "self-double emulsifying drug delivery system (SDEDDS), which are the formulated mixtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are thermodynamically stable systems. In SDEDDS the external phase water is not added.

Unlike double emulsions, SDEDDS changed the process of second emulsification step, which can self- emulsify to w/o/w double emulsions due to the gastrointestinal peristaltic movements in-vivo instead of artificial emulsification process in-vitro. In addition, SDEDDS can be filled directly into soft or hard gelatin capsule which are easy to administration and storage.

(Qi et al., 2011) developed a novel self-double-emulsifying drug delivery systems (SDEDDS) of Pidotimod by formulating mixtures of hydrophilic surfactants and w water-in-oil (w/o) emulsions. This was done with an objective for enhancing oral bioavailability of pidotimod which belong to BCS class III i.e. high solubility and low permeability. SDEDDS can spontaneously emulsify to water-in-oil- in-water (w/o/w) double emulsions in the mixed aqueous gastrointestinal environment, with drugs encapsulated in the internal water phase of the double emulsions. The optimized pidotimod-SDEDDS were found to be have sustained release of drug and stable up to 6 months under 25◦C. Plasma-concentration–time profiles from pharmacokinetic studies in rats dosed with SDEDDS showed 2.56-fold ($p<0.05$) increased absorption of

pidotimod, compared to the pidotimod solution. Thus SDEDDS may be a promising strategy for peroral delivery of BCS class III drugs.

(Wang et al., 2015) prepared nattokinase-loaded self-double-emulsifying drug delivery system (SDEDDS) by formulating w/o emulsion using self-emulsifying oil phase (MCT and liquid paraffin) and lipophilic emulsifiers (Abil EM90, Arlacel P135 and Span80) and finally proportionally mixing w/o emulsion with Hydrophilic emulsifier (Labrasol) under magnetic stirring. They showed that SDEDDS could successfully self-emulsify into water /oil/water multiple emulsions upon dilution in dispersion medium with gentle stirring, provide sustained release and effectively protect nattokinase activity in gastric environment.

(Hu et al., 2015) developed a novel SDEDDS of (-) Epigallocatechin-3-gallate (EGCG) belonging to BCS class III drug with low oral bioavailability. Various formulations of SDEDDS were formulated using Oil phase (MCT, GMR, Glycerol, and PGPR) and Hydrophilic surfactant (Tween 80) by two step emulsification process. The optimized formulation was found stable over a period of 6 months under intermediate condition and showed retarded release of drug with higher apparent permeability coefficient on Caco-2 monolayers compared to EGCG solution. Pharmacokinetic studies in rats revealed that solid SDEDDS showed 1.93-fold absorption of EGCG, compared to the same oral dose (500 mg/kg) of the EGCG solution.

(Bhattacharjee et al., 2017) developed a novel SDEDDS of Atenolol belonging to BCS class III drug with low oral bioavailability. The formulation was prepared by utilizing concept of two-step emulsification method, using 0.018 g tartaric acid and 50 mg atenolol in 0.1g water as aqueous phase, required amount of captex 355 and oleic acid (3:1) was taken as oil phase composition, span 80 as lipophilic emulsifier and tween 80 as hydrophilic emulsifier. They applied a 3(2) full factorial design for optimization procedure and found that optimized formulation exhibited increase in permeability as compared to the atenolol in tartaric acid solution aqueous solution.

(Qiang et al., 2018) formulated and evaluated topical hydrogels containing vitamin C-loaded self-double-emulsifying drug delivery system (SDEDDS) for topical application. The SDEDDS was formulated using evening prime rose as continuous oily phase containing PGPR (8%w/w) as hydrophobic emulsifier and Tween 80 as Hydrophilic emulsifier by two step emulsification process. Finally, hydrogels of vitamin C SDEDDS are prepared and tested for evaluation parameters. The prepared hydrogels depicted a sustained drug release over a 8 h period, enhance retention in skin and permeation through skin.

Rationale of study

Rationale 1:

Incorporation of radionuclides leads to disastrous health effects, in case of incorporation the radionuclides are transported across mucous membranes or they are injected or absorbed through skin or wound. Once they are incorporated, they are more difficult to remove. Thus the treatment involves reducing the absorption and internal deposition and enhancing the excretion of absorbed radionuclides.

Prussian blue insoluble, ferric (III) hexacyanoferrate (II), is a well-known drug for Cs-137 contamination. After oral ingestion, it is not absorbed through the intact gastrointestinal wall. Its clearance from the body depends on the gastrointestinal tract transit time. Prussian blue insoluble acts by ion-exchange, adsorption, and mechanical trapping within the crystal structure **(Thompson & Callen, 2001).** It has a very high affinity for radioactive and non-radioactive cesium and thallium. Prussian blue insoluble binds cesium and thallium isotopes in the gastrointestinal tract after these isotopes are ingested or excreted in the bile by the liver thereby reducing gastrointestinal reabsorption (enterohepatic circulation). Since the metabolism of these ions follow entero-enteric cycle, the exact site of action of PB is intestine where it inhibits reabsorption of ions and thereby increases fecal excretion. This significantly reduces biological half-life of Cs and Tl.

The only marketed formulation of PB is "Radiogardase-CS" in capsule dosage form which is a conventional formulation releasing the drug in stomach and the availability of drug at its site of action i.e. intestine gets delayed.

Thus, in order to avoid loss of drug and achieve better efficiency, a PB dosage form which should release the drug as a pulse only in intestine is planned.

Rationale 2

- All isotopes of Actinide series elements like americium, plutonium and curium are radioactive and have the potential to be harmful, leading to serious health effects.
- The only currently available chelating agents for decorporation of transuranic actinides are "Ca-DTPA and Zn-DTPA".

- DTPA, which belongs to BCS class III with oral bioavailability <3% shows low absorption through the intestine **(Stradling et al. 1993; Taylor et al. 2007; Durbin 2008; Abergel et al. 2010)**
- Therefore, a novel formulation design is intended to develop orally effective formulation of Ca- and Zn-DTPA for decorporation for improving patient compliance, while dealing mass population during emergency.

Objectives

- To develop and characterize a PB dosage form which should release the drug in duodenum for decorporation.
- To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vitro/ in-vivo* efficacy of developed formulation.

Plan of work

A. Matrix tablet of Prussian blue

1. Development and validation of UV spectrophotometric method of drug.
2. Formulation and optimization of matrix tablet
    - Hardness
    - Friability
    - Weight variation
    - Drug content
    - *In-vitro* dissolution
3. *In-vitro* adsorption efficiency study for Cs by AAS
4. Stability studies
5. Pharmacoscintigraphy studies
6. Efficacy studies and its comparison to Commercially available formulation "Radiogardase-Cs"
7. *In-vivo* safety studies
8. Statistical Analysis

B. Orally effective formulation of Ca-DTPA

1. Development and validation of UV spectrophotometric and HPLC method of drug.
2. Construction of pseudoternary phase diagrams
3. Formulation and characterization of SDEDDS
    - Viscosity analysis
    - Assessment of self-double-emulsification performance of SDEDDS formulations
    - Emulsion droplet size analysis
    - *In-vitro* drug release
    - *In-vitro* permeation studies

4. Stability studies
5. Pharmacoscintigraphy studies
6. Efficacy studies and its comparison to Commercially available formulation "Pentetate calcium trisodium injection 1000 mg "
7. *In-vivo* absorption and pharmacokinetics studies
8. Statistical Analysis

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Research student
Vaishali Agrawal

13.5.19
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Research Supervisor
Dr. A.K. Singh

DATE: 13/5/19

PLACE: New Delhi

Progress Report

Formulation Development of Various Agents to Improve its Efficiency as Internal Decorporation Agent

Vaishali Agrawal

(Research Scholar)

Registration No.: 33/Ph.D./DPSRU/2018

Supervisors

Dr. D.P. Pathak

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REPORT OF THE DOCTORAL STUDENT RESEARCH COMMITTEE (DSRC)

Name of the Research Scholar: **Vaishali Agrawal**

Registration no.: **33/Ph.D./DPSRU/2018**

Conduct of the Research Scholar: Very Good

Overall Assessment of the progress and specific comments of the Supervisor:

Presentation of the Scholar was found satisfactory. She explained all the queries raised during Presentation

Prof. S.R. Wakode
Expert

Could not attend
Prof. S.P. Agrawal
Expert

Dr. Vibhav Garg
Expert

Prof. D.P. Pathak
(Supervisor)
Director, DIPSAR

Dr. A.K.Singh
(Supervisor)
Outstanding Scientist
&DG (LS)

Dr.Nidhi Sandal
(Co-Supervisor)
Sc. E, INMAS

Date: 03.12.19

Annexure A: Title and objective for approval
Annexure B: Progress report from date 07-09-2019 to 06-09-2019
Annexure C: Executive summary
Annexure D: Brief Review
Annexure E: List of Publications (Communicated)

Annexure A

Title: Formulation development of various agents to improve its efficiency as internal decorporation agent

Objectives

The objective of this study is to develop formulations of various agents to improve its efficiency as internal decorporation agent. The specific objectives aimed in the present work are as follows

- To develop and characterize a PB dosage form which should release the drug in duodenum for decorporation.
- To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vitro/ in-vivo* efficacy of developed formulation

Vaishali Agrawal
Name/Signature of Research Scholar

3.12.19
Supervisor:
Dr. D.P. Pathak
Director(DIPSAR)

Supervisor:
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Outstanding Scientist & DG, LS
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Annexure B

PROGRESS REPORT FROM Date: 07-03-2019 to 06-09-2019

1. **Name of Research Scholar:** Vaishali Agrawal
2. **Correspondence Address:** Institute of Nuclear Medicine and Allied Sciences, Delhi-110054
3. **Contact No:** 9808851957
4. **Email:** agrawal.vaishali911990@gmail.com
5. **Registration No.:**33/PhD/DPSRU/2018
6. **Date of Registration:** 07-09-2018
7. **Semester Fee deposited**:** Rs 30385/- Dt.26-02-2018
8. **Approved Topic of research:**Formulation development of various agents to improve its efficiency as internal decorporation agent
9. **Nature of Fellowship, if any:** DRDO Fellowship (JRF)
10. **Details of leaves availed during the period under report, if any:**Copy attached
11. ***Research work done during the period:** Copy of detailed report attached
12. **Research papers:**
    a) Agrawal, V., Baghel, R., Singh, A. K., Pathak, D. P., & Sandal, N. (2019). Development and Validation of LC–MS Method for the Estimation of N-Acetyl-Tryptophan and its Impurities Under Stress Conditions. *Chromatographia*, *82*(10), 1467-1477.
    b) Patent Filed entitled **"pH dependent release pharmaceutical formulation and process for its preparation thereof"**, accorded **"201913044367"** as application number.
13. *** Any other information:** Copy attached

- Awarded cash prize for Oral presentation on **"Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"**in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
- Poster presentation on **"Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"**in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.

Vaishali Agrawal
ignature of Research scholar)

Dr. D.P. Pathak
Signature of Supervisor

Dr. A.K. Singh
Signature of Supervi

Date:

Annexure C

EXECUTIVE SUMMARY

A nuclear disaster during military, industrial, or medical practices leads to release of radionuclides which results in external and/or internal contamination with radioactive materials. Internal contamination occurs when radioactive materials have ingested, inhaled, or penetrated through skin or wound. After internalization, radionuclides are distributed to various tissues with patterns depending on the chemical and physical form of the radio nuclide. The pattern of injury is decided by the tissue distribution of a radionuclide and health status of exposed individuals. It is required to remove the radionuclide from GIT and systemic circulation by using surface active adsorbent or chelating agents. Decorporating agent is a broad term that includes all agents that can either prevent the incorporation of the radionuclides or decorporate the incorporated radionuclides. These agents are categorized as blocking agents, chelating agents, diluting agents and ion exchanger. The decorporating agents can be used for prevention of radionuclides absorption from g.i.t to systemic circulation, hastening the elimination of absorbed radionuclides from blood stream or decorporation of radionuclides from their target organs.

There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Potassium iodide for decontamination of iodine Calcium- and Zinc-diethylenetriaminepentaacetic acid (Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium). Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions, having high efficiency at high pH and site of action is duodenum (small intestine). Ca-DTPA is known for removal of actinides like plutonium, americium, curium and other bivalent ions. But no formulation is available in India. Thus these two decorporating agents were selected and their oral dosage form development needs to be carried out.

The available market formulation of PB i.e. "Radiogardase-Cs" is a conventional dosage form, having high dose. In one approach, modified oral dosage form of PB is designed with matrix tablet, having pH dependent release only in duodenum preventing entero-hepatic circulation of cesium radionuclide and minimization of dose. Various parameters of matrix tablet will be evaluated like hardness, friability, drug content, *in-vitro* adsorption studies,

acoscintigraphy studies, *in-vivo* efficacy studies its comparison to Commercially ble formulation "Radiogardase-Cs and i*n-vivo* safety studies.

since, the only currently available chelating agents for decorporation of transuranic ides are "Ca-DTPA and Zn-DTPA", available as i.v. DTPA has low oral bioavailability %. Therefore, in another approach, the oral formulation of Ca-DTPA is designed for orporation for improving patient compliance, while dealing mass population during ergency. One of the possible formulation of **"SDEDDS"** which are the formulated xtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are ermodynamically stable systems found to have great potential for BCS class III drugs, hancing their oral bioavailability**.** Various parameters of SDEDDS formulation will be aluated like viscosity analysis, emulsion droplet analysis, *in-vitro* drug release, *in-vitro* ermeation studies, efficacy studies and its comparison to Commercially available ormulation "Pentetate calcium trisodium injection 1000 mg , *In-vivo* absorption and harmacokinetics studies. **In conclusion** the present study will explore the potential of matrix tablet of *Prussian* blue for the removal of cesium radionuclides with its target release in duodenum and reduction of dose. Secondly SDEDDS and other approaches for enhancing oral bioavailability will be explored for Ca-DTPA. Both formulations will be optimized in order to enhance better decorporation efficiency for target radionuclides.

Annexure D

BRIEF REVIEW

There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Calcium- and Zinc-diethylenetriaminepentaacetic acid **(Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer)** for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium).

Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions. It is a crystal lattice that exchanges potassium for cesium at the surface of crystal **(Thompson & Callen, 2004).** It has been used both in animals **(R. Barroso-Moguel et al., 1994)** and clinically in humans **(Pau, 2000).** Radioactive cesium (^{137}Cs) is the most harmful naturally occurring radionuclide, with a long half-life (30.17 years) and high water solubility and mobility, which readily enters the animal and human food chains through the consumption of contaminated water, plants, meat, fish, and milk **(Ansoborlo & Wayne, 2015).** Moreover, Cs in animals and humans is processed pharmacokinetically in the same way as sodium (Na) and potassium (K) owing to its chemical analogy with those elements **(Chen et al., 2013)**. The ability and ion–exchange property of Prussian blue plays an important role in trapping heavy metal ions and enhancing their faecal excretion **(Reddy et al., 1996, Schwudke et al., 2000, Zakharchuk, 1999).** After the 1987 contamination incident in Goiania, Brazil, Prussian blue was used successfully, reducing whole-body committed radiation doses by up to a factor of **two (Melo et al., 1996; Farina & Brandiio-Mello, 1991)** . The efficacy of PB has been reported at high pH.

The only currently available chelating agents for decorporation of transuranic actinides are **"Ca-DTPA and Zn-DTPA".** They forms stable chelates with metal ions by exchanging calcium and zinc respectively for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. Due to its charge and hydrophilicity, DTPA shows poor oral bioavailability (approximately 3%) **(Volf, 1984).**

Annexure E

Publications

Agrawal, V., Baghel, R., Singh, A. K., Pathak, D. P., & Sandal, N. (2019). Development and Validation of LC–MS Method for the Estimation of N-Acetyl-Tryptophan and its Impurities Under Stress Conditions. *Chromatographia*, 82(10), 1467-1477.

- Awarded cash prize for Oral presentation on "Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
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- Patent Filed entitled **"pH dependent release pharmaceutical formulation and process for its preparation thereof"**, accorded **"201913044367"** as application number.

Doctoral Student Research Committee (DSRC)

SECOND MEETING

Period: 07-03-2019 to 06-09-2019

Ph.D. Research Scholar: Vaishali Agrawal

Registration no.: 33/Ph.D./DPSRU/2018

Date of Enrolment: 26/02/2018

Date of Registration: 07/09/2018

Topic: Formulation Development of Various Agents to Improve its Efficiency as Internal Decorporation Agent

Supervisors

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Doctoral Student Research Committee (DSRC)

SECOND MEETING

Period: 07-03-2019 to 06-09-2019

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Introduction:

Accidents involving nuclear reactors, industrial sources, or medical sources leads to internal radioactive contamination **(USFDA, 2006).** The different modes of internalization of radionuclides may be inhalation, ingestion, injection or absorption through intact or abraded skin. After internalization, radionuclides are distributed to various tissues with patterns depending on the chemical and physical form of the radio nuclide. The pattern of injury is decided by the tissue distribution of a radionuclide and health status of exposed individuals **(Cassatt et al., 2008)** The retention time of radioactive contaminants in the body decide the extent of significant health risk pose to the body. Thus the removal of such radioactive contaminants is the only method to reduce such health risks **(USFDA, 2006)**.

The aim of internal decorporation is to reduce absorption and enhance excretion of radioactive contaminants. Decorporation agents refers to drug products that increase the rate of elimination or excretion of absorbed, inhaled, or ingested radioactive contaminants **(USFDA, 2006)**. Decorporating agents is a broad term that may either prevent incorporation of radionuclide or decorporate the incorporated radionuclide. These agents may be categorized as blocking agents, chelating agents, diluting agents, ion exchanger. Decorporation agents can be used at three levels:

1. Prevention of radionuclides absorption from git to systemic circulation.
2. Hastening the elimination of absorbed radionuclides from blood stream.
3. Decorporation of radionuclides from target organs.

Removal of internalized radionuclide is a vast problem due to: Large number of radionuclides, their different chemical form and solubility, physical decay, multiple route of entry, bio kinetics and organ specificity. Complete removal of internalized radionuclide from body is rather impossible, thus we intend to develop a ready to use clinically validated decorporation agents formulated in desired dosage form which can be used by military people for decorporation of internalized radionuclides. The radioisotopes, their chemical form and their amount cannot be predicted, therefore decorporation agents that can provide maximum protection were used. There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Potassium iodide for decontamination of iodine Calcium- and Zinc-diethylenetriaminepentaacetic acid (Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals,

Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium). Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions and Ca-DTPA for removal of actinides like plutonium, americium, curium and other bivalent ions. Thus these two decorporating agents were selected and their oral dosage form development needs to be carried out.

Prussian blue (Ferric ferrocyanide) is a dye used for internal decorporation of Cs-137 and ^{201}Tl. It is a crystal lattice that exchanges potassium for cesium at the surface of crystal **(Thompson & Callen, 2004).** It has been used both in animals **(R. Barroso-Moguel et al., 1994)** and clinically in humans **(Pau, 2000).** Radioactive cesium (^{137}Cs) is the most harmful naturally occurring radionuclide, with a long half-life (30.17 years) and high water solubility and mobility, which readily enters the animal and human food chains through the consumption of contaminated water, plants, meat, fish, and milk **(Ansoborlo & Wayne, 2015).** Moreover, Cs in animals and humans is processed pharmacokinetically in the same way as sodium (Na) and potassium (K) owing to its chemical analogy with those elements **(Chen et al., 2013)**. Approximately 10% of Cs is eliminated rapidly with a biological half-life of 2 days, 90% is eliminated gradually with a biological half-life of 110 days, and less than 1% remains with a longer biological half-life of approximately 500 days15. These type of insoluble contaminants target the gastrointestinal (GI) tract traveling the entire length of the tract unabsorbed and excrete via hepatobiliary clearance. Some nonabsorbed binding resins find utility in inhibiting the uptake of radioactive contaminants in the gut. E.g. Prussian blue after oral intake, not absorbed by the GI tract, binds cesium ions in the gut enhancing its fecal excretion. After the 1987 contamination incident in Goiania, Brazil, Prussian blue was used successfully, reducing whole-body committed radiation doses by up to a factor of **two (Melo et al., 1996; Farina & Brandiio-Mello, 1991) .**

The efficacy of PB has been reported at high pH. Therefore the purpose of the study is to target the drug at duodenum of small intestine and also avoid overdosing of PB.

Ca-DTPA and Zn-DTPA forms stable chelates with metal ions by exchanging calcium and zinc respectively for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. The chelating capacity of Ca-DTPA is greatest immediately and approximately 24 hours after internal contamination when the radio contaminants are still circulating and readily available for chelation. After the first dose of Ca-DTPA, maintenance dose of CA-DTPA or Zn-DTPA resulted in similar rates of elimination but Ca-DTPA was found to be associated with greater toxicity, including more depletion of trace

metals, higher rate of mortality, presence of kidney and liver vacuolization, and small bowel hemorrhagic lesions. The recommended initial dose of Ca-DTPA in adults and adolescents is 1 g administered intravenously. In patients younger than 12 years of age, a single initial dose of 14 mg/kg (not to exceed 1 g) should be administered intravenously. These salts are not found to be effective for decorporation of uranium, neptunium or radioactive iodine **(Cada, 2005).**

Both Ca-DTPA and Zn-DTPA are poorly absorbed following oral administration. DTPA is not very effective when administered orally possibly due to the low absorption of DTPA through the intestines. It belongs to BCS class III category. It has very low oral bioavailability **(approx. 3%) (Abergel, 2016; Durbin, 2008; Taylor, Hodgson, & Stradling, 2007).**

Therefore the purpose of the study is to develop the oral formulation of Ca-DTPA in order to improve patient compliance and ease of administration. One such design may be the formulation of "self-double emulsifying drug delivery system (SDEDDS), which are the formulated mixtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are thermodynamically stable systems found to have great potential for BCS class III drugs, enhancing their oral bioavailability **(Qi et al., 2011).**

Rationale of study

Rationale 1:

Incorporation of radionuclides leads to disastrous health effects, in case of incorporation the radionuclides are transported across mucous membranes or they are injected or absorbed through skin or wound. Once they are incorporated, they are more difficult to remove. Thus the treatment involves reducing the absorption and internal deposition and enhancing the excretion of absorbed radionuclides.

Prussian blue insoluble, ferric (III) hexacyanoferrate (II), is a well-known drug for Cs-137 contamination. After oral ingestion, it is not absorbed through the intact gastrointestinal wall. Its clearance from the body depends on the gastrointestinal tract transit time. Prussian blue insoluble acts by ion-exchange, adsorption, and mechanical trapping within the crystal structure **(Thompson & Callen, 2001).** It has a very high affinity for radioactive and non-radioactive cesium and thallium. Prussian blue insoluble binds cesium and thallium isotopes in the gastrointestinal tract after these isotopes are ingested or excreted in the bile by the liver thereby reducing gastrointestinal reabsorption (enterohepatic circulation). Since the metabolism of these ions follow entero-enteric cycle, the exact site of action of PB is intestine where it inhibits reabsorption of ions and thereby increases fecal excretion. This significantly reduces biological half-life of Cs and Tl.

The only marketed formulation of PB is "Radiogardase-CS" in capsule dosage form which is a conventional formulation releasing the drug in stomach and the availability of drug at its site of action i.e. intestine gets delayed.

Thus, in order to avoid loss of drug and achieve better efficiency, a PB dosage form which should release the drug as a pulse only in intestine is planned.

Rationale 2

- All isotopes of Actinide series elements like americium, plutonium and curium are radioactive and have the potential to be harmful, leading to serious health effects.
- The only currently available chelating agents for decorporation of transuranic actinides are "Ca-DTPA and Zn-DTPA".

- DTPA, which belongs to BCS class III with oral bioavailability <3% shows low absorption through the intestine **(Stradling et al. 1993; Taylor et al. 2007; Durbin 2008; Abergel et al. 2010)**
- Therefore, a novel formulation design is intended to develop orally effective formulation of Ca- and Zn-DTPA for decorporation for improving patient compliance, while dealing mass population during emergency.

Objectives

- To develop and characterize a PB dosage form which should release the drug in duodenum for decorporation.
- To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vitro/ in-vivo* efficacy of developed formulation.

Experimental Methods

➢ Development and Characterization of pH dependent release dosage form

A. Development and Validation of UV spectrophotometric method of Prussian blue

Samples were analysed UV spectrophotometrically by moistening 0.01 g of sample material with 1 ml water followed by addition of 1 ml of 1 N sodium hydroxide with continuous shaking. A reddish brown precipitate was formed within 10 minutes of incubation. Thereafter, 0.2 ml of conc. Sulphuric acid is added to above solution; an intense blue colour solution appears. The solution was scanned using UV spectrophotometer (Perkin Elmer, L365) in the range of 400 nm to 1000 nm.

B. Spectral Analysis

Infrared spectroscopy is an important analytical tool to ascertain the identity of a chemical compound. PB is particularly attractive for infrared studies since the system contains the strongly absorbing CN functional group.

C. Preparation and optimization of Prussian blue granules

Different combinations of granules as per Table 1 were prepared using wet granulation technique. Optimization of granules was done based on its in-vitro release behavior of drug in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

Table 1: Formulation design of Prussian blue granules

S.No.	Code	Prussian blue (g)	Sodium alginate (%w/w)	Calcium chloride (%w/w)	Calcium gluconate (%w/w)	MCC (%w/w)
1	G1	1	5	1.5	-	-
2	G2	1	5	2	-	-
3	G3	1	5	2.5	-	-

4	G4	1	7.5	2	-	-
5	G5	1	7.5	2.5		-
6	G6	1	5	-	2	-
7	G6	1	5	-	5	-
8	G7	1	5	-	10	-
9	G8	1	5	1.5	5	10
10	G9	1	5	1.5	5	15
11	G10	1	5	1.5	5	20
12	G11	1	5	5	5	20

D. Pre-compressional evaluation of granules

The optimized granules were subjected for pre compressional evaluation by determining the bulk density, tapped density, compressibility index, Hausners ratio, moisture analysis and flow properties (angle of repose) by using standard procedures. All studies were carried out in triplicate (n=3) and average values are re*p*orted.

E. Formulation and Development of pH dependent Matrix release tablets of Prussian blue

The optimized granule composition was further selected for tablet compression. The weighed mixture of drug and polymers was sufficiently moistened with required quantity of PVP K30 in water (10%w/v). The dough so formed was allowed to pass through mesh size #12 and the wet granules were dried in tray drier at 60º C for 45-60 min. Further the dried granules were passed through mesh size #14 for uniform size of the granules followed by addition of extra-granular ingredients. The granules were compressed to form tablets using a single station automatic tablet punching machine (lab press; Neel Equipment Technology, Ahmedabad, India) with 10-mm punches. The average weight of the tablets was kept constant for all formulation combinations (500 mg). The formulation design is mentioned in Table 2.

Table 2: Formulation design of Prussian blue matrix tablet

S.No.	Tablet Co De	Amount of PB (mg)	Sodium alginate (%w/w)	Calcium Gluconate (%w/w)	Calcium chloride (%w/w)	MCC (%w/w)	Talc (%w/w)	Mg. stearate (%w/w)	PVP k30 (%w/w)
1.	F1	500	5	5	5	20	3	1.5	10

F. In process Quality Control of Prussian blue Tablet

All prepared tablets (F1) were evaluated for their Uniformity of weight, Hardness, Friability, Drug content and Drug release according to the Indian Pharmacopeia (IP).

F.1 Tablet Hardness

The tablet crushing strength was tested by the commonly used Pfizer-type tablet hardness tester (IEC, Mumbai, India). Five tablets were randomly selected; each tablet was placed between the anvils and the crushing strength, which causes the tablet to break and average values were recorded.

F.2 Tablet Friability

This test is generally applicable to mostly compressed tablets and supplements other physical strength measurements. It is conducted to evaluate the ability of the tablets to withstand the breakage during the transportation and handling. As per USP, sample of whole tablets corresponding to 6.5 g were taken, dedusted prior to testing, accurately weighed the tablet sample and placed the tablets in the drum of Tablet friability test apparatus; Roche Friabilator (LABINDIA, Delhi, India). The drum was rotated 100 times, and tablets were removed, dedusted and final weight was taken. The percent weight loss was calculated as per the formula

$$\%F = (Wi - Wf)/ Wi \times 100$$

Where

% F: friability in percentage; W_i: initial weight of tablets; W_f : final weight of tablets after 100 revolutions.

F.3 Tablet Weight Variation

Twenty tablets were randomly selected and weighed individually. The average weight was calculated. It was ensured that not more than two of the individual tablet weights deviated from the average weight by more than 5% and none deviated by more than twice that percentage.

F.4 Drug Content Determination

Five tablets were randomly selected, weighed, and crushed. 100mg powder was subjected to acid-base treatment and after dilution with water, estimated by UV spectrophotometric method at λ_{max} = 710 nm .A tablet is said to comply with the test if individual drug content is between 85 and 115% of the average content (label claim). On the other hand, a preparation fails to comply with the test if more than one tablet content is outside these limits or if any individual content is outside the limits as mentioned in IP-2010.

F.5 Drug Release Study

In vitro drug-release studies was carried out in triplicate, in the dissolution test apparatus (USP Type I; Dissolution test apparatus, LABINDIA) as per International Conference on Harmonization (ICH) guidelines. The basket rotation was set to a speed of 75 rpm. The tablets were placed into 500 ml of deaerated simulated gastric fluid (pH 1.2) for 2 h maintained at 37°C after which the dissolution media was changed to 500 mL phosphate buffer (pH 6.8). Sampling was done at predetermined time intervals (5, 10,20,30,60 min) and assayed spectrophotometrically at a 710 nm as mentioned in previous section. Cumulative percent drug release with time (h) was determined.

Further pH dependent release of Prussian blue was studied in citrate-phosphate buffers of pH 3, 4, 5, 6, and 7. F1 was subjected to dissolution studies as described above and concentration of PB was determined.

F.6 Analysis of Release Mechanism

The release mechanism of PB from optimized matrix formulation was examined in accordance with the kinetic models. The regression coefficient, value nearer to 1.0, indicated the model fitting of the release mechanism. The commonly adopted models for understanding the release of drugs from matrices, namely, zero-order equation, first-order equation, Higuchi equation, Hixson-Crowell equation and Koresmeyer-Peppas simple exponential equation were used to elucidate the mode of release.

G. Maximum binding capacity of Prussian blue

Maximum binding capacity of Prussian blue was estimated for Cesium and Thallium with the help of Atomic absorption spectroscopy. Cesium chloride and Thallium chloride were taken as standards for estimation by AAS. 50 mL of 600, 750, 900, 1200 or 1500 ppm Cesium stock solutions and 50 mL of 100,200,400 or 600 ppm Thallium stock solutions were added to a 100-mL glass flask, followed by the addition of 0.1 g of PB sample. The flask was tightly stopped and incubated in a shaking water bath at 37 ◦C with 75 shakes/min for 24 h. Upon completion of incubation, sample was filtered with a 0.2 _m Acrodisc® syringe filter (Gelman Laboratory, MI), and then 10mL aliquot of sample was transferred to a glass test tube. The sample was diluted with 5% nitric acid and analyzed by AAS. All samples were prepared in triplicate.

H. Pharmacoscintigraphy Evaluation

H.1 *In-vitro* evaluation

Sodium alginate was tagged in Prussian blue matrix formulation with pertechnetate ions and allowed to get dried at room temperature for 2h. Tagging was confirmed by Paper chromatography, calculating Top and Bottom counts and % tagging was calculated.

H.2 Release Profile of Labeled Dosage Forms

In vitro release profile of radiolabeled dosage form was determined by measuring radioactive counts released at regular time intervals as used in in-vitro drug release study. 0.1 N HCl was used as dissolution media (Scientific Systems 8S) for first 2 h followed by phosphate buffer of pH 6.8. The study was carried out at 75 rpm and 37°C. 1 ml samples were withdrawn and measured for radioactive counts in gamma ray spectrometer (ECIL, India). The release pattern of activity was compared to the drug release.

H. In-vivo Pharmacoscintigraphy Evaluation

Sodium alginate was tagged in the same way as discussed in section H.1. This study was done in **Rabbit Model.** Imaging was done using Gamma camera, at predetermines time intervals up to 24 h.

H.3 Pharmacoscintigraphy Evaluation

In-vivo pH-dependent release of formulation was studied in Rabbit animal model by gamma scintigraphy using barium chloride as a tracer compound. The study protocol was approved by the Institutional Animal Ethics Committee duly constituted for the purpose vide letter no. **INM/IAEC/2018/07.**

- **Development and characterization of orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.**

A. Development and Validation of UV spectrophotometric method of Ca-DTPA

UV spectroscopic method was developed by ion-pairing reaction of Ca-DTPA with Ferric chloride and scanned using UV spectrophotometer (Perkin Elmer, L365) in the range of 200 nm to 400 nm.

B. In-vitro Permeation study of Ca-DTPA

Membrane diffusion and permeability studies were performed with Franz diffusion cell method A vertical Franz diffusion cell system (**Orchid Scientific, Nashik).** 0.5 of sample was placed as a donor phase on PVDF membrane (the pore diameter was 0.22 μm, Millipore). Before use, membrane was allowed to soak in Millipore water for 20 min. The effective diffusion surface area was 3.4 cm2. Millipore water was used in receptor compartment. The rotation of the magnetic stir bar was set to 200 rpm. Experiments were performed at 37±0.5 °C. The permeation was examined for 4 h. Samples of 1.0 ml were taken from the receptor phase manually and replaced with fresh receiving medium. The quantitative measurement of Ca-DTPA was carried out with an UV spectrophotometer at a wavelength of λ=280 nm. The mean values (±SD) were calculated from three parallel measurements each time.

Results

➢ Development and Characterization of pH dependent release dosage form

A. Development and Validation of UV spectrophotometric method of Prussian blue

UV spectroscopic method of Prussian blue was developed and validated as per ICH guidelines with the help of various parameters like Accuracy, Precision, Linearity, LOD and LOQ. The results of validation parameters are given below in Table 2 and Fig.1

Table 2: Validation parameters of UV spectroscopy of Prussian blue

Parameters	Value
Linearity (Range, µg/mL)	10-100
Regression coefficient	y = 0.0192x + 0.0148 R^2 = 0.9968
Accuracy	97.1-98.3
Precision	<2%
LOD (µg/mL)	6.05
LOQ(µg/mL)	18.33

A.

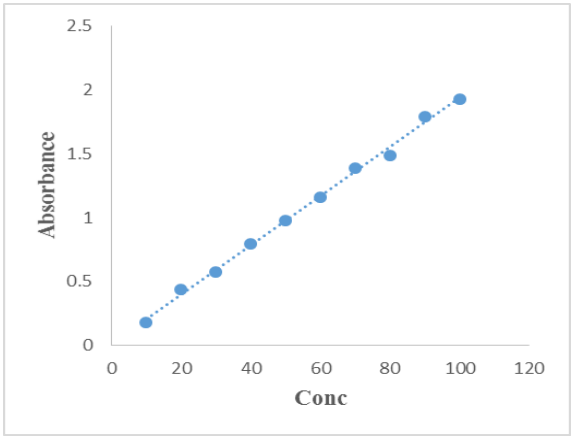


Fig. 1 Calibration curve of Prussian blue

B. Spectral Analysis

The FT-IR spectra of Prussian blue showed several peaks, Fig.2, the strong peak at 2057cm-1 which is the characteristic absorption peak of PB is assigned as the stretching vibration of C≡N group.

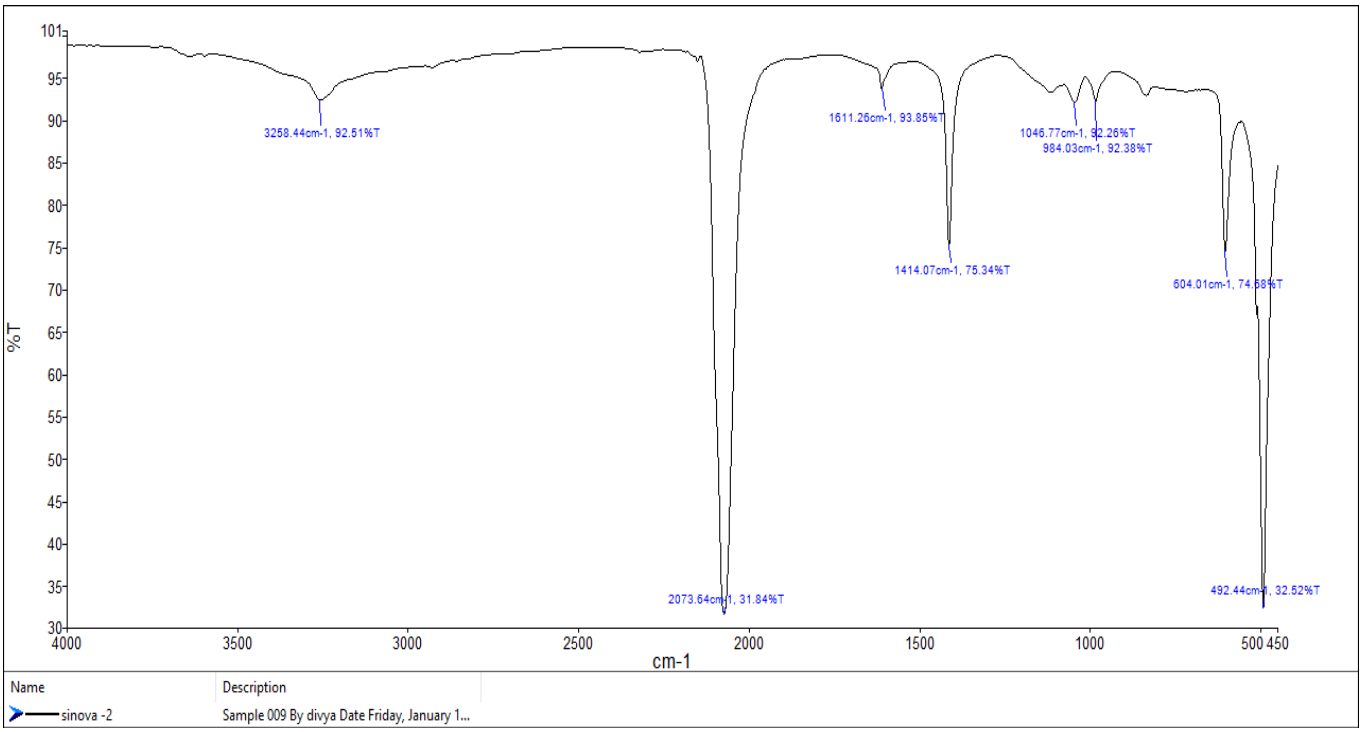


Fig.2 Infrared Spectra of Prussian blue

The absorption bands near 3258.44 and 1611 cm-1 is assigned to the O–H stretching mode and H–O–H bending mode respectively, which indicate the existence of interstitial forces of attraction in the sample. The absorption bands around 604 cm^{-1}, are due to the structure of Fe2+ -CN-Fe3+ linkage of Prussian blue

C. Formulation and Development of pH dependent Matrix release tablets of Prussian blue

Matrix tablets of Prussian blue was formulated through wet granulation technique using Sodium alginate as a pH sensitive polymer and binder, Calcium gluconate as a source of $Ca^{2+,}$ Calcium chloride as a disintegrant as well as source of Ca^{2+}, Microcrystalline cellulose due to its compressibility properties along with extra granular ingredients like Magnesium stearate as a lubricant and talc as glidant. The formulation design is mentioned in Table 1.

D. In process Quality Control of Prussian blue Tablet

The prepared tablets (F1) were evaluated for physical characteristics like tablet hardness, friability, weight variation, drug content and *in vitro* drug release. The hardness of the tablets was measured by a Pfizer-type tablet hardness tester (IEC, Mumbai, India) was found to be 5.3 ± 0.28 kgs. The friability was below 1% for all the formulations. Weight variation in different formulations was found to be 434.5 to 505.03 mg. The percentage of drug content for different

batches was found to be 75±2%. The results of *in-vitro* dissolution studies of F1 are shown in Fig. 3. Further pH dependent release was also studied in citrate-phosphate buffer pH range (3-7) and the results are shown in Fig.4.

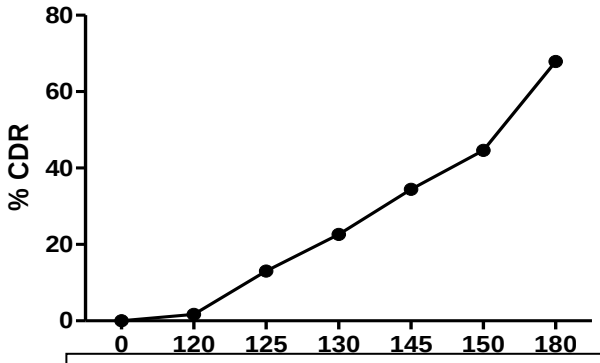


Fig.3 In-vitro dissolution profile of Matrix release tablet of Prussian blue (F1)

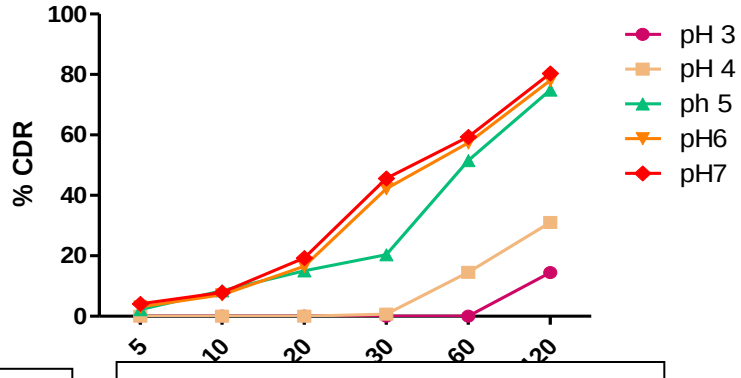


Fig. 4 pH dependent release study of F1 at various pH buffer range (3-7)

The release data obtained from in-vitro release study was subjected to various simple exponential equation models to elucidate the mode of drug release. These included zero-order equation, first-order equation, Hixson-crowell, and Koresmeyer–Peppas simple exponential equation models. The slope and regression coefficient (R^2) are shown in Table 3 and Fig. 5. The formulation fitted best for Hixson-crowell equation with an R^2 value of 0.9906.

Table 3 Kinetic release data for different models of PB matrix formulation

Kinetic Model	Slope	Regression coefficient
Zero-order	62.49	0.9612
First-order	1.18	0.587
Hixson-crowell	**1.89**	**0.9906**
Koresmeyer–Peppas	0.090	0.6324

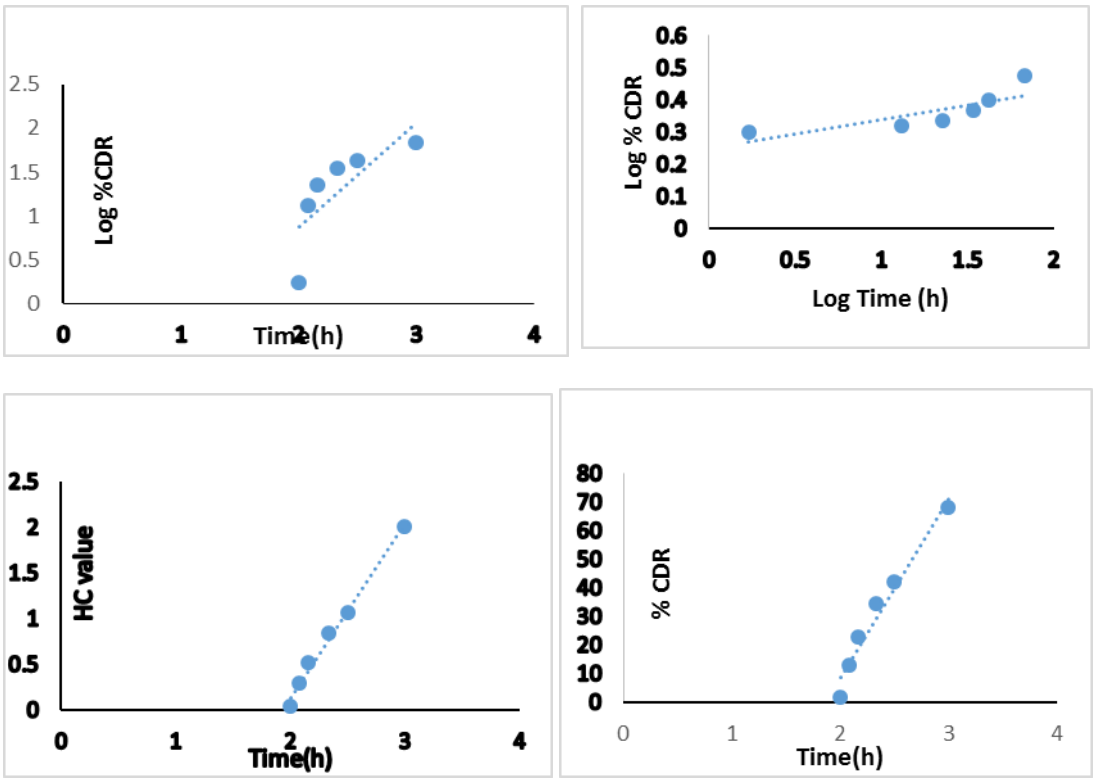


Fig. 5 Comparative plot of various exponential equation models fitted to PB matrix tablet release a) First order b) Koresmeyer-Peppas model c) Hixson crowell model d) Zero order

E. Maximum binding capacity of Prussian blue

The maximal binding capacity of PB to cesium and Thallium was determined by taking binding data from the five cesium concentrations (600–1500 ppm) and Four Thallium concentrations (100-600ppm) were used. Binding data of 24 h incubation for both cesium and thallium was used for plotting the Langmuir isotherm According to the Langmuir equation:

$$q_e = (q_{max} * b * C_e)/(1+b*C_e);$$

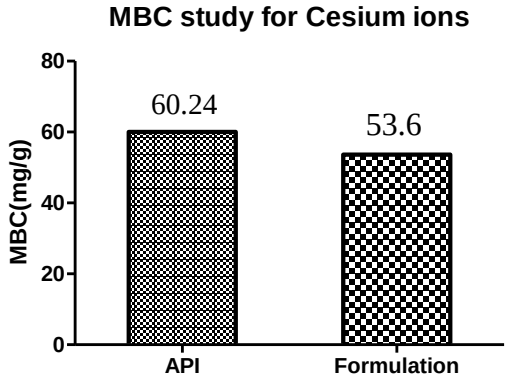
q_e is defined as mass of metal removed (mg) per mass of adsorbent used (g) (dry weight)

C_e is the metal ion concentration at equilibrium (mg/L),

q_{max} is maximum metal removal capacity (mg/g), and b Langmuir adsorption constant.

Where equation can be written as: $C_e/q_e = C_e/q_{max} + 1/q_{max}*b$ where, slope $=1/q_{max}$ and intercept $= 1/q_{max}*$

The Results of MBC of both API and Formulation are shown in Fig.6 and Fig.7.



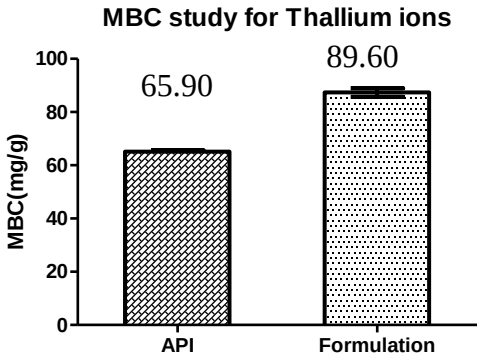


Fig.6 Comparative MBC of API and F1 for Cesium ions

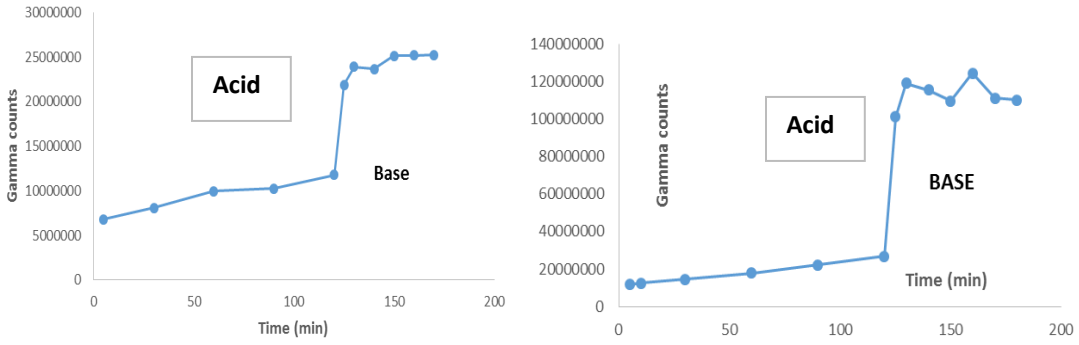
Fig.7 Comparative MBC of API and F1 for Thallium ions

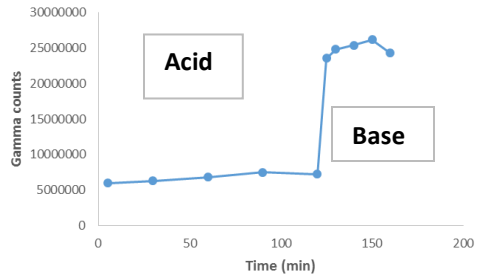
F. Pharmacoscintigraphy evaluation

F.1 In-vitro evaluation

Prussian blue granules containing sodium alginate was tagged with pertechnetate ions, allowed to dried at room temperature for 2 h. The in-vitro release study was performed with tagged granule and the release pattern of activity was compared to drug release. The results are mentioned below:

Fig. 8 In-vitro relase gamma counts of Matrix formulation





F.2 In-vivo Pharmacoscintigraphy Evaluation

This study was conducted in Rabbit Model using tagged Sodium alginate containing Prussian blue matrix formulation. Static imaging was done using Gamma camera, at predetermined time intervals up to 24 h. The result is mentioned in Fig.9.

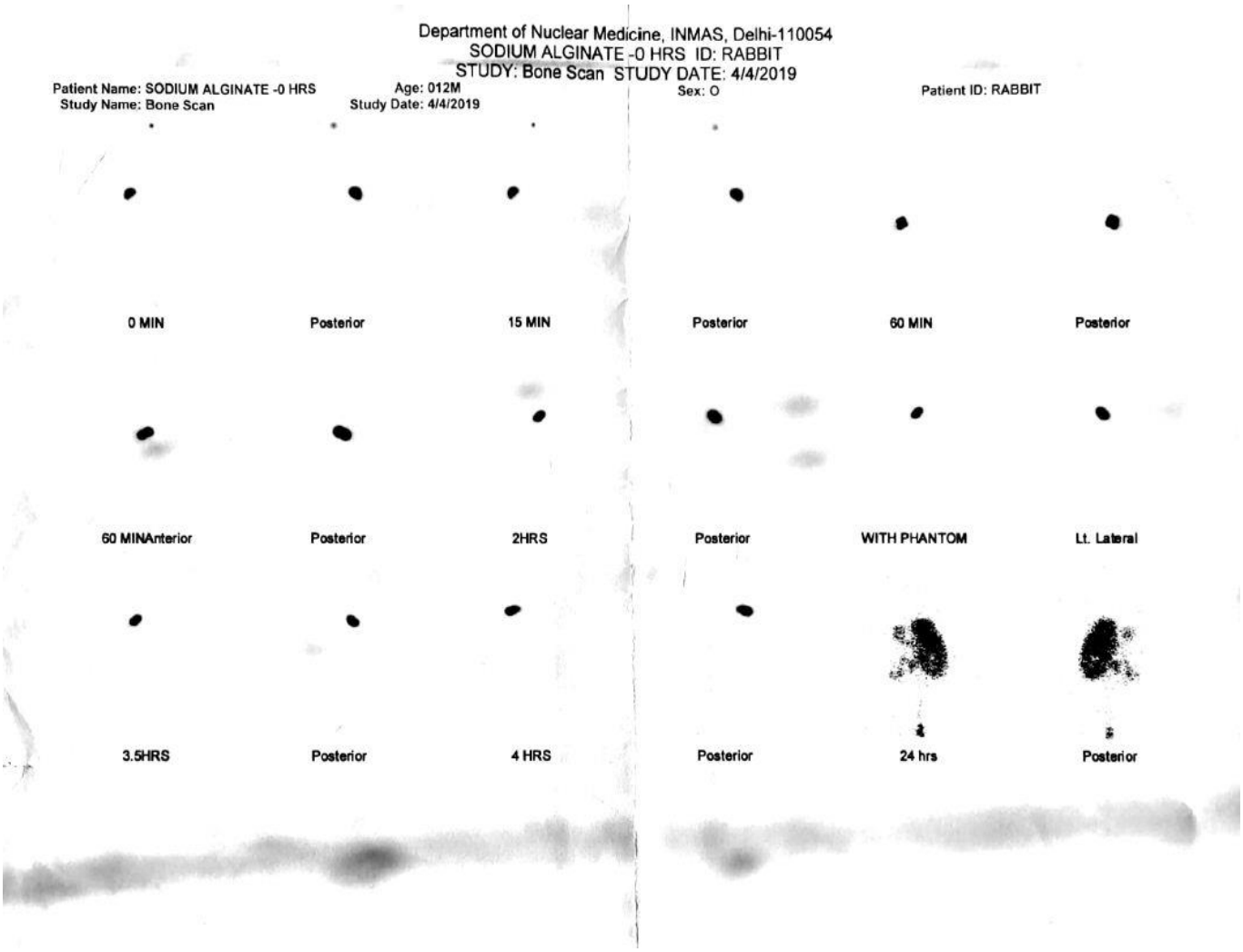


Fig. 9 Static images of Rabbit after oral administration of tagged Prussian blue granule

F.3 *In-vivo* Pharmacoscintigraphy Evaluation.

Since, Tagging with pertechnetate ions did not act as true tracer as shown above. Thus, Nuclear medicine doctors suggested "**Barium chloride"** to be used in experimentation **as** a true tracer. Control treatment with barium chloride dosing only; while treatment model exposed to Prussian blue formulation containing 1% of Barium chloride. Static imaging was done using "Gamma **Camera**" at prefixed time intervals and "**Hounsfield** unit (**HU**)" values were observed (Table 4).

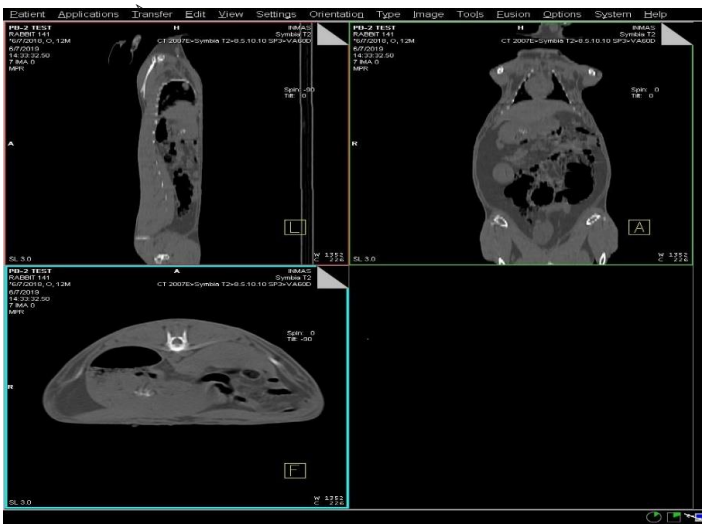
Table 4: HU (**Hounsfield unit**) values observed during CT imaging of rabbit.

Time Points	Stomach	Intestine
Control	146,177,202,103,155	289,438,356,547,242,250,238
Test (0 h (Initial)	408	248
	404	213
	219	132
	169	278
	155	234
Test (2h)	470	257
	496	131
	202	201
	123	171
	130	167
Test (4h)	377	208
	435	227
	164	275
	274	185
	207	232

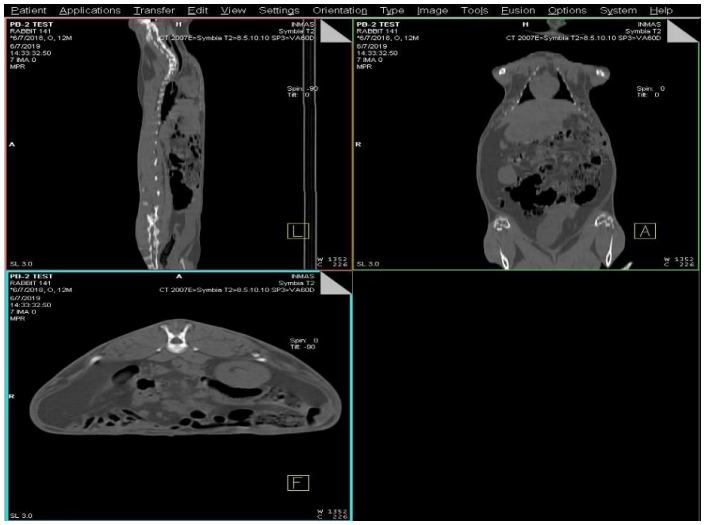
Fig. 10 (a) Control group

10. (b) Stomach section (0h)

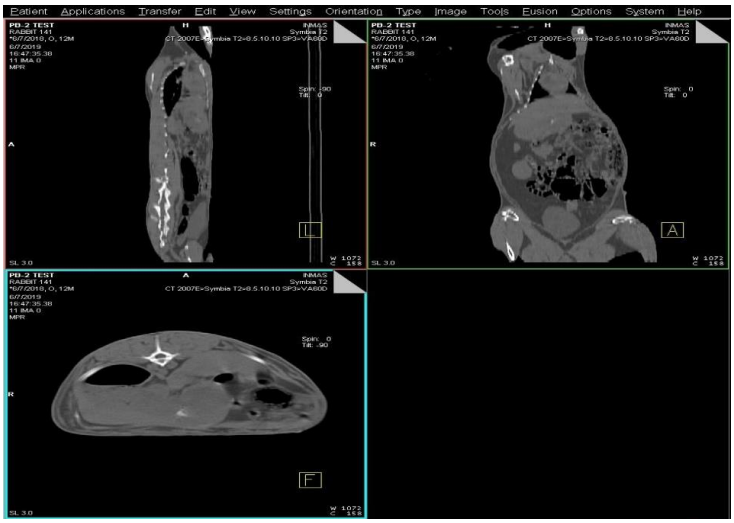
Intestine section (0h)



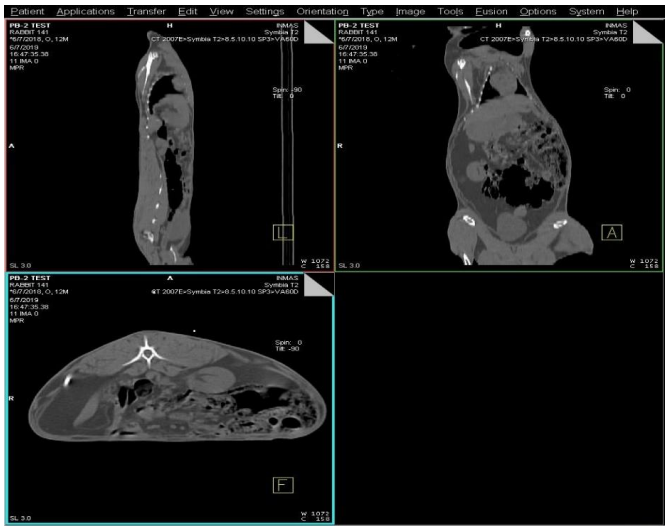
Stomach section (2h)



Intestine section (2h)



Stomach section (4h)



Intestine section (4h)

- **Development and characterization of orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.**

A. Development and Validation of UV spectrophotometric method of Ca-DTPA

UV spectroscopic method of Ca-DTPA was developed using ion-pairing reaction with Ferric chloride and the validation was done as per ICH guidelines. The results are mentioned below in Fig. 11 and Table 4.

Linearity (Range, µg/mL)	10-100 Y= 0.0129x-0.0086
Regression coefficient	0.9998
Accuracy (% Recovery)	97.1-98.3
Precision (%RSD)	< 2%
LOD(µg/mL)	1.6
LOQ(µg/mL)	4.8

Table 4: Validation parameters of UV spectroscopic method of Ca-DTPA

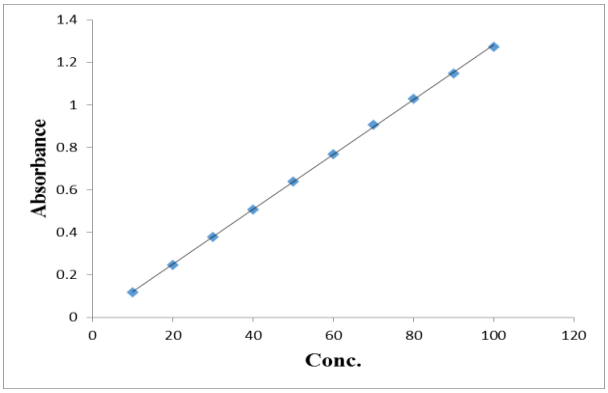
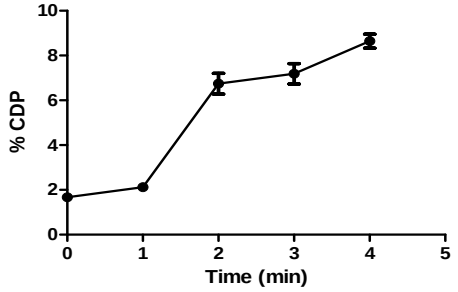


Fig.11 Calibration curve of Ca-DTPA

B. In-vitro permeation study of Ca-DTPA

The membrane permeation studies were conducted using Franz diffusion apparatus with the help of Durapore PVDF membrane (pore size, 0.22 micrometer). The study was conducted for 4 h and percentage cumulative permeation of drug through barrier was calculated and plotted with respect to time (**Fig.12a)** and % Cumulative drug permeated per unit area was also plotted **(Fig. 12b**).



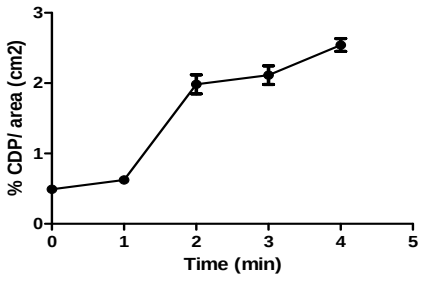


Fig. 12 (a) % CDP v/s time plot of Ca-DTPA 12(b) % CDP/area v/s time plot of Ca- DTPA

Discussion

A. Prussian blue is a well-known decorporation agent for removal of Cs-137 and ^{201}Tl. It is a crystal lattice that exchanges potassium for cesium at the surface of crystal **(Thompson & Callen, 2004).** Cesium follows the movement of potassium and is excreted into the intestine, reabsorbed from the gut into the blood, then to the bile, where it is excreted again into the gut (enterohepatic circulation) **(Radiogardase, FDA).** Thus the present study was aimed to formulate matrix formulation of Prussian blue which can target cesium and thallium ions entering in to the gut through enterohepatic circulation and facilitates its excretion. The Matrix tablets were formulated through wet granulation technique using Sodium alginate as a pH sensitive and hydrophilic matrix forming polymer and binder, Calcium gluconate as a source of Ca^{2+}, Calcium chloride as a disintegrant as well as source of Ca^{2+}, Microcrystalline cellulose due to its compressibility properties along with extra granular ingredients like Magnesium stearate as a lubricant and talc as glidant. Various in process quality control parameters of F1 as per I.P was done like hardness, friability, weight variation, drug content and in-vitro drug release. All tablets in a batch was found to contain optimum hardness, friability in limits and found to comply with weight variation test. Further the results of *in-vitro* drug release showed that the formulation did not release drug in stomach (pH 1.5) but once it comes in contact with high pH (6.8) in intestine it releases drug through erosion. Thus Calcium alginate behave as an erodible matrix which at high pH eroded to release PB. Free study alginate when comes in contact with the acidic stomach environment, as a result of hydrogen bonding, it alternates into insoluble alginic acid, which controls water penetration and prevents matrix disintegration and further when it gets cross linked with divalent metal ions such as Ca^2 it further improve the mechanical strength of alginate polymer and reduces its solubility in water. **(Szekalska et al., 2018).** Calcium ions from calcium gluconate and calcium chloride helps in creating cross linked structure with alginate polymer and helps in sustaining the drug in acidic conditions. Thus application of a cross-linking agent leads to a reduction in the solubility of the polymer matrix under acidic conditions, which could be an effective approach to sustain the release of freely water-soluble drugs. At higher pH, calcium alginate matrix tablets get swollen and undergoes erosion due to break down of gel structure. (Mandal) Subsequent placement of the alginate matrix tablets in a solution of higher pH may lead to reconversion of alginic acid to o determined for both cesium and thallium ions.

B. UV spectroscopic method of Ca DTPA was developed and validated using ion-pairing reaction with Ferric chloride. *In-vitro* experimentation study was conducted with pure drug and found that the drug showed permeation only up to 10% when passed through membrane filter (pore size 0.22 micrometer).

Conclusion

- pH dependent matrix release tablets of Prussian blue was successfully developed and characterized for their release in intestine. Maximum binding capacity study showed the binding capacity of Prussian blue per gram for both cesium and thallium ions.
- UV spectroscopic method of Ca-DTPA was developed and validated as per ICH guidelines. Further *in-vitro* permeation study was conducted and the results showed up to 10% permeation.

Future Plans

- Stability Studies
- Pharmacoscintigraphy studies
- Efficacy studies and its comparison to Commercially available formulation "Radiogardase-Cs"
- In-vivo safety studies
- Development and validation of HPLC method of Ca-DTPA
- Development of orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- Development and characterization of orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vivo* efficacy of developed formulation.

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Research student
Vaishali Agrawal

Research Supervisor
Dr. D.P. Pathak

Research Supervisor
Dr. A.K. Singh

DATE:

PLACE: New Delhi

Doctoral Student Research Committee (DSRC)

THIRD MEETING

Period: 07-09-2019 to 24-09-2020

Ph.D. Research Scholar: Vaishali Agrawal

Registration no.: 33/Ph.D./DPSRU/2018

Date of Enrolment: 26/02/2018

Date of Registration: 07/09/2018

Topic: Formulation Development of Various Agents to Improve its Efficiency as Internal Decorporation Agent

Supervisors

Dr. D.P. Pathak

Dr. A.K. Singh

Co-Supervisor

Dr. Nidhi Sandal

DIPSAR

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...ORT OF THE DOCTORAL STUDENT RESEARCH COMMITTEE (DSRC)

...me of the Research Scholar: Vaishali Agrawal
Registration no.: 33/Ph.D./DIPSRU/2018
Conduct of the Research Scholar: Satisfactory

Overall Assessment of the progress and specific comments of the Supervisor:

Presentation of the scholar was virtual. It was satisfactory. She answered all the questions raised by the experts.

Prof. S.R. Wakode
Expert

Could not attend
Prof. S.P. Agrawal
Expert

Vibhav Garg
Dr. Vibhav Garg
Expert

Prof. D.P. Pathak
(Supervisor)
Director, DIPSAR

Dr. A.K. Singh
(Supervisor)
Outstanding Scientist
&DG (LS)

Dr.Nidhi Sandal
(Co-Supervisor)
Sc. E, INMAS

Date: 24-09-20

Annexure A: Title and objective for approval
Annexure B: Progress report from date 07-09-2019 to 24-09-20
Annexure C: Executive summary
Annexure D: Brief Review
Annexure E: List of Publications

Annexure A

Title: Formulation development of various agents to improve its efficiency as internal decorporation agent

Objectives

The objective of this study is to develop formulations of various agents to improve its efficiency as internal decorporation agent. The specific objectives aimed in the present work are as follows

- To develop and characterize a pH dependent release PB dosage form to improve its efficacy as internal decorporation agent
- To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vivo* efficacy of developed formulations.

Vaishali Agrawal
Name/Signature of Research Scholar

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Dr. D.P. Pathak
Director(DIPSAR)

Supervisor:
Dr. A.K. Singh
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DRDO

Co-Supervisor

Dr.Nidhi Sandal
Sc.E, INMAS

Annexure B

PROGRESS REPORT FROM Date: 07-09-2019 to 24-09-2020

1. **Name of Research Scholar:** Vaishali Agrawal
2. **Correspondence Address:** Institute of Nuclear Medicine and Allied Sciences, Delhi-110054
3. **Contact No:** 9808851957
4. **Email:** agrawal.vaishali911990@gmail.com
5. **Registration No.:**33/PhD/DPSRU/2018
6. **Date of Registration:**07-09-2018
7. **Semester Fee deposited**:** Rs 30385/- Dt.26-02-2018
8. **Approved Topic of research:**Formulation development of various agents to improve its efficiency as internal decorporation agent
9. **Nature of Fellowship, if any:** DRDO Fellowship (SRF)
10. **Details of leaves availed during the period under report, if any:**Copy attached
11. ***Research work done during the period:** Copy of detailed report attached
12. **Research papers:**
    a) Agrawal, V., Baghel, R., Singh, A. K., Pathak, D. P., & Sandal, N. (2019). Development and Validation of LC–MS Method for the Estimation of N-Acetyl-Tryptophan and its Impurities Under Stress Conditions. *Chromatographia*, *82*(10), 1467-1477.
    b) Patent Filed entitled**"pH dependent release pharmaceutical formulation and process for its preparation thereof",** accorded **"201913044367"** as application number.
    c) pH dependent release matrix oral formulation of prussian blue to improve its efficacy as internal decorporation agent (under Review in Current drug delivery).
13. *** Any other information:** Copy attached

- Awarded cash prize for Oral presentation on **"Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"**in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
- Poster presentation on **"Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"**in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.

Vaishali Agrawal
(Signature of Research scholar)

Dr.D.P. Pathak
Signature of Supervisor

Dr. A.K. Singh
Signature of Super

Dr.Nidhi Sandal
Signature of Co-Supervi

Date: 24-09-20

Annexure C

Title: Formulation development of various agents to improve its efficiency as internal decorporation agent

Executive Summary:

A nuclear disaster during military, industrial, or medical practices leads to release of radionuclides which results in external and/or internal contamination with radioactive materials. Decorporating agents are used that can be used for prevention of radionuclides absorption from g.i.t to systemic circulation, hastening the elimination of absorbed radionuclides from blood stream or decorporation of radionuclides from their target organs. These agents are categorized as blocking agents, chelating agents, diluting agents and ion exchanger.There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Potassium iodide for decontamination of iodine Calcium- and Zinc-diethylenetriaminepentaacetic acid (Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium).

Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions, having high efficiency at high pH and site of action is duodenum (small intestine). The available market formulation of PB i.e. "Radiogardase-Cs" is a conventional dosage form, having high dose (not available in India). Therefore with agenda of minimization of dose and target release in duodenum, the present study aims to formulate matrix formulation of Prussian blue which can target cesium and thallium ions entering in to the gut through enterohepatic circulation and facilitates their excretion. The matrix release tablet formulation of Prussian blue was developed and validated for various in process quality control parameters like hardness, friability, weight variation, drug content and *in-vitro* drug release. All tablets in a batch was found to contain optimum hardness (5.3 ± 0.28 kgs), friability in limits(<1%), drug content (75±2%) and found to comply with weight variation test (434.5 to 505.03 mg). Further the results of *in-vitro* drug release showed that the formulation did not release drug in stomach (pH 1.5) but once it comes in contact with high pH (6.8) in intestine it releases complete drug within 30-60 min.The formulation was found to comply Hixson-crowell model with r2 value (0.9906) thus confirming erosion dependent release mechanism. pH dependent release study was also done in citrate-phosphate buffer (pH 3-7) and found that significant release in intestinal pH starts at pH 5 or more. Further, the maximal binding capacity of PB to Thallium

was determined by taking binding data from four thallium concentrations (100-600ppm). Binding data of 24 h incubation for thallium was used for plotting the Langmuir isotherm. The MBC of formulation and API for thallium was found to be 89.6 and 65.90 mg/g of PB respectively.The proof of concept i.e. drug release in duodenum was studied through pharmacoscintigraphy using radio labeled formulation in rabbits. *In-vitro* pharmacoscintigraphy studies was done using surface tagging of optimized formulation with perrtechnetate and *in-vitro* release study was done. The samples were analyzed through Gamma counter and the release pattern of activity was compared to drug release. This also confirms the same pattern of release i.e. minimal release in gastric pH and complete release in intestinal pH. Therefore, Calcium alginate here behave as an erodible matrix which is able to retain drug at acidic pH and eroded to release PB at high pH. *In-vivo* Pharmacoscintigraphy studies was done in rabbits and pharmacoscintigraphic images confirmed intact formulation in stomach up to 2h and burst release in intestine thereafter.

Ca-DTPA is known for removal of actinides like plutonium, americium, curium and other bivalent ions. Ca-DTPA has low oral bioavailability, thus available as i.v (not available in India). Therefore, the oral formulation of Ca-DTPA is designed as decorporation agent for improving patient compliance, while dealing mass population during emergency. Ca-DTPA did not contain chromophores thus UV spectroscopic method was developed by ion-pairing reaction of Ca-DTPA with Ferric chloride and validated based on various parameters like Linearity, Accuracy, Precision, LOD and LOQ as per ICH guidelines. HPLC method of Ca-DTPA was developed and validated for various parameters as per ICH guidelines. Further, *in-vitro* permeation studies of Ca-DTPA were carried out with the help of Durapore PVDF membrane (pore size, 0.22 micrometre) using Franz diffusion apparatus. The study was conducted for 4 h, samples were analysed through HPLC and percentage cumulative permeation of drug through barrier was calculated and plotted with respect to time. The drug showed permeation only up to 0.6%.. With the purpose to enhance oral bioavailability of Ca-DTPA, one of the possible formulation isSelf-double emulsifying drug delivery system (SDEDDS) which are the formulated isotropic mixtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are thermodynamically stable systems found to have great potential for BCS class III drugs, enhancing their oral bioavailability. Various excipients for formulating SDEDDS were screened i.e. castor oil, olive oil, corn oil, medium chain triglycerides, Tween 80, Labrasol, Span 60, Span 80 etc. The solubility studies of Ca-DTPA in each vehicle was conducted using shake flask method and the oil with minimum solubility

Annexure C

MCT was selected for formulating SDEDDS system. Oleic acid was also added as it was reported to enhance bioavailability. Various formulations with variable concentration range of excipients are under trial development phase and optimized on the basis of various parameters like viscosity analysis, emulsion droplet analysis, *in-vitro* drug release, *in-vitro* permeation studies. Further efficacy studies and its comparison to Commercially available formulation "Pentetate calcium trisodium injection 1000 mg , *In-vivo* absorption and pharmacokinetics studies was also conducted.

In conclusion the present study explored the potential of matrix tablet of Prussian blue for the removal of thallium radionuclides with its target release in duodenum and reduction of dose. Secondly SDEDDS and other approaches for enhancing oral bioavailability will be explored for Ca-DTPA. Both formulations will be optimized in order to enhance better decorporation efficiency for target radionuclides.

Vaishali Agrawal

Research scholar

DPSRU, INMAS (DRDO)

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Annexure D

BRIEF REVIEW

There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)**for decontamination of cesium, Calcium- and Zinc-diethylenetriaminepentaacetic acid **(Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer)** for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium).

Prussian blue is well known for removal of monovalent Cs^+/Tl^+ ions. `It is a crystal lattice that exchanges potassium for cesium at the surface of crystal **(Thompson & Callen, 2004).** It has been used both in animals **(R. Barroso-Moguel et al., 1994)**and clinically in humans **(Pau, 2000).** Radioactive cesium (^{137}Cs) is the most harmful naturally occurring radionuclide, with a long half-life (30.17 years) and high water solubility and mobility, which readily enters the animal and human food chains through the consumption of contaminated water, plants, meat, fish, and milk **(Ansoborlo & Wayne, 2015).** Moreover, Cs in animals and humans is processed pharmacokinetically in the same way as sodium (Na) and potassium (K) owing to its chemical analogy with those elements **(Chen et al., 2013)**.The ability and ion–exchange property of Prussian blue plays an important role in trapping heavy metal ionsand enhancing their faecal excretion **(Reddy et al., 1996, Schwudke et al., 2000, Zakharchuk, 1999).** After the 1987 contamination incident in Goiania, Brazil, Prussian blue was used successfully, reducing whole-body committed radiation doses by up to a factor of **two (Melo et al., 1996; Farina &Brandiio-Mello, 1991).**The efficacy of PB has been reported at high pH.

The only currently available chelating agents for decorporation of transuranic actinides are **"Ca-DTPA and Zn-DTPA".** They forms stable chelates with metal ions by exchanging calcium and zinc respectively for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. Due to its charge and hydrophilicity, DTPA shows poor oral bioavailability (approximately 3%) **(Volf, 1984).**

Annexure E

List of Publications

- Agrawal, V., Baghel, R., Singh, A. K., Pathak, D. P., & Sandal, N. (2019). Development and Validation of LC–MS Method for the Estimation of N-Acetyl-Tryptophan and its Impurities Under Stress Conditions. *Chromatographia*, *82*(10), 1467-1477.
- Awarded cash prize for Oral presentation on "Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
- Poster presentation on "Development and validation of LC-MS method for the estimation of N-Acetyl- Tryptophan and its impurities under stress conditions"in 70th IPC held at Amity University, Noida on 21-23 Dec 2018.
- Patent Filed entitled **"pH dependent release pharmaceutical formulation and process for its preparation thereof",** accorded **"201913044367"** as application number.

- pH dependent release matrix oral formulation of prussian blue to improve its efficacy as internal decorporation agent (under Review in Current drug delivery).

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Topic: Formulation Development of Various Agents to Improve its Efficiency as Internal Decorporation Agent

Supervisors

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Introduction:

Accidents involving nuclear reactors, industrial sources, or medical sources leads to internal radioactive contamination [1]. The different modes of internalization of radionuclides may be inhalation, ingestion, injection or absorption through intact or abraded skin. After internalization, radionuclides are distributed to various tissues with patterns depending on the chemical and physical form of the radio nuclide. The pattern of injury is decided by the tissue distribution of a radionuclide and health status of exposed individuals [2]. The retention time of radioactive contaminants in the body decide the extent of significant health risk pose to the body. Thus the removal of such radioactive contaminants is the only method to reduce such health risks [1].

The aim of internal decorporation is to reduce absorption and enhance excretion of radioactive contaminants. Decorporation agents refers to drug products that increase the rate of elimination or excretion of absorbed, inhaled, or ingested radioactive contaminants [1]. Decorporating agents is a broad term that may either prevent incorporation of radionuclide or decorporate the incorporated radionuclide. These agents may be categorized as blocking agents, chelating agents, diluting agents, ion exchanger. Decorporation agents can be used at three levels:

1. Prevention of radionuclides absorption from git to systemic circulation.
2. Hastening the elimination of absorbed radionuclides from blood stream.
3. Decorporation of radionuclides from target organs.

Removal of internalized radionuclide is a vast problem due to: Large number of radionuclides, their different chemical form and solubility, physical decay, multiple route of entry, bio kinetics and organ specificity. Complete removal of internalized radionuclide from body is rather impossible, thus we intend to develop a ready to use clinically validated decorporation agents formulated in desired dosage form which can be used by military people for decorporation of internalized radionuclides. The radioisotopes, their chemical form and their amount cannot be predicted, therefore decorporation agents that can provide maximum protection were used. There are few agents approved by the U.S. Food and Drug Administration (FDA) for decorporation i.e. Prussian blue capsules **(Radiogardase, 500 mg capsules, Heyl, Berlin, Germany)** for decontamination of cesium, Potassium iodide for decontamination of iodine Calcium- and Zinc-diethylenetriaminepentaacetic acid (Ca and Zn-DTPA, 0.5g vials Hameln Pharmaceuticals, Hameln, Germany), administered intravenously (i.v.) or by nebulizer, for decontamination of transuranic radionuclides (e.g. plutonium, americium and curium). Prussian blue is well known

for removal of monovalent Cs^+/Tl^+ ions and Ca-DTPA for removal of actinides like plutonium, americium, curium and other bivalent ions. Thus these two decorporating agents were selected and their oral dosage form development needs to be carried out.

Prussian blue (Ferric ferrocyanide) is a dye used for internal decorporation of Cs-137 and ^{201}Tl. It is a crystal lattice that exchanges potassium for cesium at the surface of crystal [3]. It has been used both in animals [4] and clinically in humans [5]. Radioactive cesium (^{137}Cs) is the most harmful naturally occurring radionuclide, with a long half-life (30.17 years) and high water solubility and mobility, which readily enters the animal and human food chains through the consumption of contaminated water, plants, meat, fish, and milk [6]. Moreover, Cs in animals and humans is processed pharmacokinetically in the same way as sodium (Na) and potassium (K) owing to its chemical analogy with those elements [7]. Approximately 10% of Cs is eliminated rapidly with a biological half-life of 2 days, 90% is eliminated gradually with a biological half-life of 110 days, and less than 1% remains with a longer biological half-life of approximately 500 days15. These type of insoluble contaminants target the gastrointestinal (GI) tract traveling the entire length of the tract unabsorbed and excrete via hepatobiliary clearance. Some nonabsorbed binding resins find utility in inhibiting the uptake of radioactive contaminants in the gut. E.g. Prussian blue after oral intake, not absorbed by the GI tract, binds cesium ions in the gut enhancing its fecal excretion. After the 1987 contamination incident in Goiania, Brazil, Prussian blue was used successfully, reducing whole-body committed radiation doses by up to a factor of two [8-9].

The efficacy of PB has been reported at high pH. Therefore the purpose of the study is to target the drug at duodenum of small intestine and also avoid overdosing of PB.

Ca-DTPA and Zn-DTPA forms stable chelates with metal ions by exchanging calcium and zinc respectively for a metal of greater binding capacity. Then radioactive chelates are excreted by glomerular filtration in to the urine. The chelating capacity of Ca-DTPA is greatest immediately and approximately 24 hours after internal contamination when the radio contaminants are still circulating and readily available for chelation. After the first dose of Ca-DTPA, maintenance dose of CA-DTPA or Zn-DTPA resulted in similar rates of elimination but Ca-DTPA was found to be associated with greater toxicity, including more depletion of trace metals, higher rate of mortality, presence of kidney and liver vacuolization, and small bowel hemorrhagic lesions. The recommended initial dose of Ca-DTPA in adults and adolescents is 1 g administered intravenously. In patients younger than 12 years of age, a single initial dose of 14

mg/kg (not to exceed 1 g) should be administered intravenously. These salts are not found to be effective for decorporation of uranium, neptunium or radioactive iodine [10].

Both Ca-DTPA and Zn-DTPA are poorly absorbed following oral administration. DTPA is not very effective when administered orally possibly due to the low absorption of DTPA through the intestines. It belongs to BCS class III category. It has very low oral bioavailability **(approx. 3%)** [11-13].

Therefore the purpose of the study is to develop the oral formulation of Ca-DTPA in order to improve patient compliance and ease of administration. One such design may be the formulation of "self-double emulsifying drug delivery system (SDEDDS), which are the formulated mixtures of water-in-oil (w/o) emulsions and hydrophilic surfactants. These are thermodynamically stable systems found to have great potential for BCS class III drugs, enhancing their oral bioavailability [14].

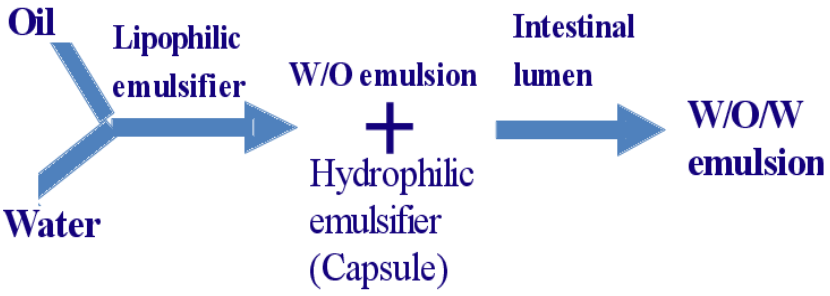

Fig.1 Schematic diagram of formation of SDEDDS system

Rationale of study

Rationale 1:

Incorporation of radionuclides leads to disastrous health effects, in case of incorporation the radionuclides are transported across mucous membranes or they are injected or absorbed through skin or wound. Once they are incorporated, they are more difficult to remove. Thus the treatment involves reducing the absorption and internal deposition and enhancing the excretion of absorbed radionuclides.

Prussian blue insoluble, ferric (III) hexacyanoferrate (II), is a well-known drug for Cs-137 contamination. After oral ingestion, it is not absorbed through the intact gastrointestinal wall. Its clearance from the body depends on the gastrointestinal tract transit time. Prussian blue insoluble acts by ion-exchange, adsorption, and mechanical trapping within the crystal structure [3]. It has a very high affinity for radioactive and non-radioactive cesium and thallium. Prussian blue insoluble binds cesium and thallium isotopes in the gastrointestinal tract after these isotopes are ingested or excreted in the bile by the liver thereby reducing gastrointestinal reabsorption (enterohepatic circulation). Since the metabolism of these ions follow entero-enteric cycle, the exact site of action of PB is intestine where it inhibits reabsorption of ions and thereby increases fecal excretion. This significantly reduces biological half-life of Cs and Tl.

The only marketed formulation of PB is "Radiogardase-CS" in capsule dosage form which is a conventional formulation releasing the drug in stomach and the availability of drug at its site of action i.e. intestine gets delayed.

Thus, in order to avoid loss of drug and achieve better efficiency, a PB dosage form which should release the drug as a pulse only in intestine is planned.

Rationale 2

- All isotopes of Actinide series elements like americium, plutonium and curium are radioactive and have the potential to be harmful, leading to serious health effects.
- The only currently available chelating agents for decorporation of transuranic actinides are "Ca-DTPA and Zn-DTPA".
- DTPA, which belongs to BCS class III with oral bioavailability <3% shows low absorption through the intestine [11-13].

- Therefore, a novel formulation design is intended to develop orally effective formulation of Ca- and Zn-DTPA for decorporation for improving patient compliance, while dealing mass population during emergency.

Objectives

- To develop and characterize a pH dependent release PB dosage form to improve its efficacy as internal decorporation agent
- To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
- To evaluate *in-vivo* efficacy of developed formulations.

Experimental Methods

I. Develop and characterize a pH dependent release PB dosage form to improve its efficacy as internal decorporation agent

1. Estimation of Maximum binding capacity (MBC) of Prussian blue for Thallium

Thallium chloride solution of 1000 ppm was used as a standard. Stock solutions of 100, 200, 400 and 600 ppm were prepared from 1000 ppm standard solution. Standard curve was prepared in the range of 5-60 ppm and linearity was observed.

1.1 Analytical Method validation: All standards and samples were analyzed by atomic absorption spectroscopy (AAS) using a LABINDIA AA 8000 atomic absorption/emission spectrometer equipped with an air–acetylene burner. The solution was aspirated into an air–acetylene flame for a time period of 5 s. Each sample was analyzed in triplicate by AAS. Sample blanks were run between every three samples or standards to assess carry over. Thallium was detected at an emission line of 276.8 nm. The method was validated based on various parameters like precision, accuracy, linearity, range. LOD and LOQ [14].

1..2 Experimental Method: To the 50 mL Falcon, 50 mL each of 100,200,400 and 600 ppm thallium stock solutions were taken and 0.1 g PB formulation or 0.1 g PB API was added to it. The falcon was tightly stoppered and incubated in a shaking water bath at 37 ◦C for 24h. Upon completion of incubation, sample was filtered using whatman filter papers. The filtrate was analyzed using atomic absorption spectroscopy (AAS). Sample dilutions was made with 5% nitric acid. All samples were prepared in triplicate. The MBC of PB formulation and PB API for thallium was determined by using **Langmuir adsorption isotherm** model. The Langmuir adsorption isotherm was represented by following equation

$q_e = (q_{max}*b*C_e)/(1+b*C_e)$; a

q_e is defined as mass of metal removed (mg) per mass of adsorbent (g) (dry weight); C_e is the metal ion concentration at equilibrium (mg/L);q_{max} is maximum metal removal capacity (mg/g), and b Langmuir adsorption constant.

Where equation can be written as:

$C_e/q_e = C_e/q_{max} + 1/q_{max}*b$ where, slope $=1/q_{max}$ and intercept $= 1/q_{max}*b$.

2. Pharmacoscintigraphy Evaluation

2.1 Radiolabeling of PB formulation: The optimized PB formulation was surface labeled with reduced technetium pertechnetate ($^{99m}TcO_4^-$). Technetium pertechnetate was reduced with 50 µg stannous chloride (SnCl2 4 mg/ml in 0.01 N HCl solution and pH was adjusted to 7 using 0.1 N NaOH solution. Surface was exposed to reduced activity and allowed to dry for 30 min.

2.2 In-vitro release profile of Labeled dosage form: The radiolabeled PB formulation were studied for *in-vitro* activity release as per method discussed in Sec. 2.5.2 and measured for radioactive counts using gamma ray spectrometer. The release pattern of radioactivity was observed for approximating labeling efficiency as well as compared to drug release.

2.3 In-vivo pharmacoscintigraphy: pH-dependent release of radiolabeled PB formulation was studied *in-vivo* in Rabbit animal model using gamma scintigraphy. The drug dose was reduced to 100mg in order to make feasible for rabbit administration. The study protocol was approved by the Institutional Animal Ethics Committee duly constituted for the purpose vide letter no. INM/IAEC/2018/07. The research adhered to the "Principles of Laboratory Animal Care". Four healthy rabbits of either sex were taken, two were taken as control and two were tested for formulation. Control 1 rabbit was administered 100mg radiolabeled conventional release PB formulation and another control 2 rabbit was administered reduced pertechnetate activity capsule. In test group, each rabbit was administered orally with 100mg radiolabeled test PB formulation having 250 microcurie of activity. Static imaging was done for both controls and test at an interval of 0, 1, 2 and 3h. At the end of 2h in control and 3h in test rabbits, 15 dynamic frames each of 120 sec were taken of the abdominal area to monitor GI transit of formulation. Blood pool activity of control and the test subjects was monitored with respect to GIT transit of formulation. A region of interest was marked over chest cavity and counts/sec versus time graph was plotted using Microsoft excel.

3. Statistical Analysis

The results were expressed as mean ±S.E.M. (n=3). The data were analyzed statistically using Graph Pad Prism 5.00. Statistical analysis were performed by one way Anova followed by Tukey's post-hoc test and two way ANOVA followed by Bonferroni multiple comparison test, wherever necessary. A value of $p<0.05$ was significant in all cases.

II. Development and characterization of orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.

A. Development and Validation of HPLC method of Ca-DTPA

1. Instrumentation

A Waters Alliance Model Agilent 1200 HPLC system consisting of autosampler (model) membrane degasser, quaternary gradient pump (model) and column thermostat was used for liquid chromatography method development and validation (Santa Clara, USA). The system was equipped with Hypersil Gold Thermo Scientific Sunfire ^{TM} C18 (250 cm × 4.6 mm) 5 μm column (Paisley, UK), and the detector consisted of UV/VIS operated at 260 nm. Empower Software (Version Rev B.04.03 (16)) was used for data processing and evaluation

2. Preparation of stock and sample solutions

A Ca-DTPA standard solutions was made by dissolving 10mg of Ca-DTPA with milli-Q water in 100 mL volumetric flask containing 1mL of 20mM anhydrous $Fecl_3$ solution to form stock solution of 100 μgmL^{-1}. From stock solution, various sample solutions of 2-20 μgmL^{-1} are made in milli-Q water. Sample solutions are allowed to get incubated for 30 min. before analysis.

3. Chromatographic conditions

TBA phosphate buffer solution (24mm) was used as Mobile phase A. It was prepared by mixing 7.79 mL TBA hydroxide solution (40% aqueous solution) in 500 mL milli-Q water. Finally pH was adjusted to 6.5 using phosphoric acid solution (Wang, 2016). 100% pure ACN was used as Mobile phase B. Prior to use both mobile phases were properly filtered through 0.45 µm membrane filters and sonicated for 10-15 min. for degassing. Ca-DTPA was analytically assayed by running single HPLC chromatographic run in isocratic gradient mode (60:40) for total chromatographic run time of 10 min.The detection was done at 260 nm. The injection volume was 25µL, flow rate was $1mLmin^{-1.}$ and the column temperature was controlled at 30º C.

4. Method validation

The HPLC method was validated for various parameters like specificity, linearity, range, accuracy, precision, , limit of detection (LOD), limit of quantification (LOQ) and robustness as per ICH and FDA guidelines (Q2R1 guidelines)[15].

1.1 Specificity.

It signifies the ability of developed HPLC method to discriminate between analyte and other compounds in the assay mixture. It was evaluated by injecting 10µL of standard drug solution, blank and placebo.

1.2 Linearity and Range

For estimation of linearity and range, different dilutions of stock concentration of Ca-DTPA i.e. 2-50 μgmL^{-1} was made. The retention time and AUC of Ca-DTPA-Fe^{3+} peak was analysed using HPLC. Each concentration was run in triplicates under same conditions. Calibration curve was plotted between AUC and concentration of drug and best fit line was plotted using least square regression method. The range is defined as interval between the upper and lower concentration (amounts) of an analyte in the sample including these concentrations for which suitable level of precision, accuracy and linearity has been attained.

1.3 Accuracy

The accuracy of the developed method was determined by assay of spiked samples at three levels i.e.50, 100 and 150% of test concentration (say 4 μgmL^{-1}) i.e. 6,8 and 10 μgmL^{-1}. Each solution was injected in triplicate. The AUC of drug was noted in each chromatogram and % recovery of added Ca-DTPA and % RSD was calculated for each sample.

1.4 Precision

The precision studies of the developed method was estimated by 1) Repeatability assays of 3 standard concentrations /3 replicates covering the entire range. 2) Intermediate precision i.e. by establishing the effects of random events on the precision of analytical procedure like days etc. The % RSD was calculated in both cases for evaluating repeatability results (ICH).

1.5 LOD and LOQ

The LOD and LOQ was estimated based on the standard deviation of the Reponse (σ) and the Slope (S). The detection limit was calculated from formula: ($3.3*\sigma/S$) and the quantitation

limit was calculated from formula (10*σ/S). The calibration curve was used to calculate slope (S) and standard deviation of y-intercepts of regression lines was used as standard deviation of response (ICH).

B. In-vitro Permeation study of Ca-DTPA

Membrane diffusion and permeability studies were performed with Franz diffusion cell method A vertical Franz diffusion cell system (**Orchid Scientific, Nashik).** 0.5 of sample was placed as a donor phase on PVDF membrane (the pore diameter was 0.22 µm, Millipore). Before use, membrane was allowed to soak in Millipore water for 20 min. The effective diffusion surface area was 3.4 cm2. Millipore water was used in receptor compartment. The rotation of the magnetic stir bar was set to 200 rpm. Experiments were performed at 37±0.5 °C. The permeation was examined for 4 h. Samples of 1.0 ml were taken from the receptor phase manually and replaced with fresh receiving medium. The quantitative measurement of Ca-DTPA was carried out with HPLC method. The mean values (±SD) were calculated from three parallel measurements each time..

C. Development and evaluation of CA-DTPA loaded SDEDDS

1. Screening of excipients for formulation of SDEDDS system

Various excipients like oil phase i.e. castor oil, corn oil, olive oil, oleic acid, medium chain triglycerides , surfactants i.e. Span 80, tween 80, Labrasol, span 60 were screened for optimizing the system.

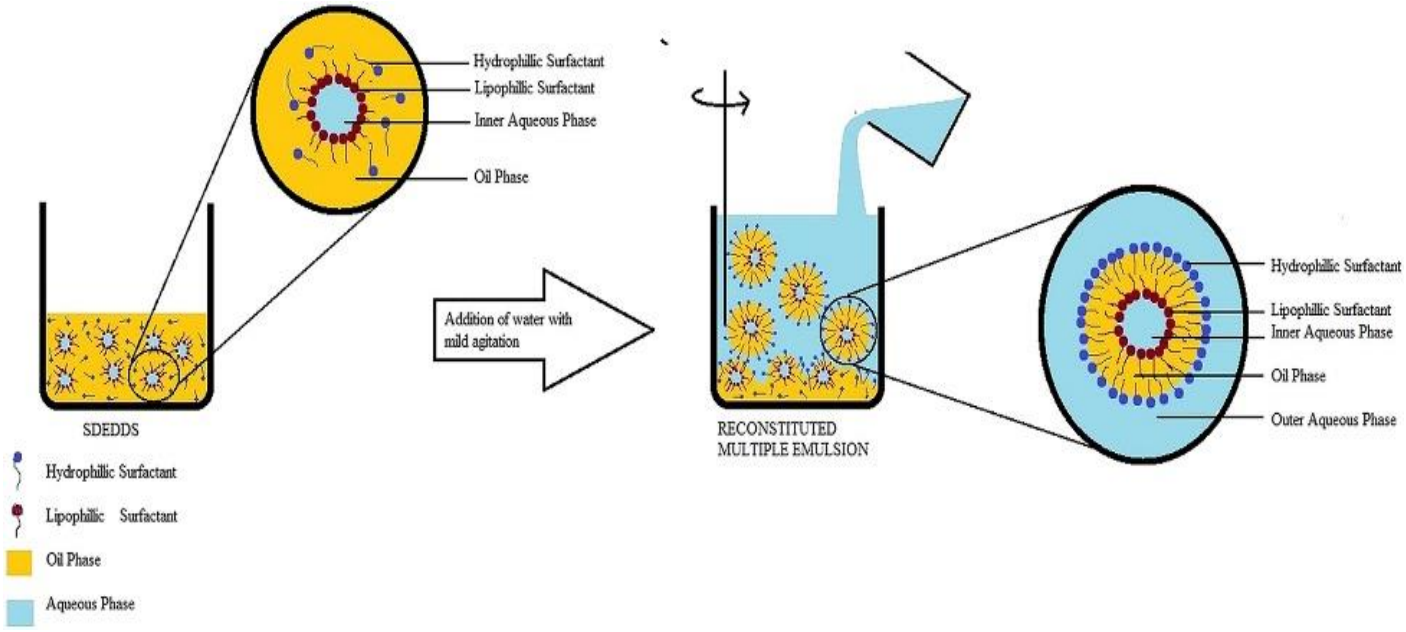

Fig. 2 SDEDDS SYSTEM

2. Soubility studies

Solubility studies were conducted using Shake flask method. 100 mg drug added with 0.5 g of each excipient and allowed to shake for 48 h in shaking water bath at 25 °C to facilitate solubilization. The samples were centrifuged at 4000 rpm for 10 min to remove undissolved drug. The Supernatant were taken and analysed using HPLC for quantification of drug.

3. Formulation and development of SDEDDS based Ca-DTPA system.

First oil phase and Lipophilic surfactants were taken in a beaker

⇩

Adding inner aqueous phase drop by drop under moderate magnetic stirring at RT

⇩

Formation of w/o primary emulsion

⇩

W/o emulsion to be homogenized at 5000 rpm for 5 min.

⇩

Then adding Hydrophilic surfactant at reduced magnetic stirring

Trial formulation design

Components category	Components taken	Conc. tried (%w/w)
Drug	Ca-DTPA(highly water soluble)	100 mg per g of emulsion
Aqueous phase	Water	10-30
Oil phase	MCT (Medium chain triglycerides) and oleic acid	MCT(40) Oleic acid (30)

Lipophilic surfactants	Span 80	10-20
Hydrophilic surfactants	Tween 80 or labrasol	10

3.1 Parameters screened

3.1.1 **Visual observation** : The developed system was visualized for appearance before dilution and after dilution.

3.1.2 **Optical microscopy**: Eosin was encapsulated and observed for globules under inverted microscope at 10X.

Results

- **Development and Characterization of pH dependent release dosage form**

1. *Maximum binding capacity for thallium*

1.1 Analytical method validation: The Atomic absorption spectroscopy method was validated as per ICH guidelines. The curve was found to be linear over the analytical range 5-60 ppm of thallium. The linear equation was found to be y=0.0092x+ 0.0193. The accuracy of the quality control standards at the low QC, intermediate QC and high QC each met the acceptance criteria of <15%. The precision of the quality control standards each met the acceptance criteria of <2%. The LOD and LOQ was found to be 0.168 and 0.5661 ppm, respectively.

1. 2 Experimental results: Langmuir Adsorption Isotherm model was used to evaluate the MBC of F3 and PB API for thallium**.** The binding data of 24 h incubation for thallium was used to draw the best fit linear graph between free thallium (Ce) v/s to PB bound thallium (Ce/qe). The MBC was calculated as inverse of slope (Fig. **3**). The MBC of F3 and PB API for thallium was found to be 89.60 and 65.90 mg/g of PB respectively. Statistically significant difference was found between value of API and F3 at $p<0.05$.

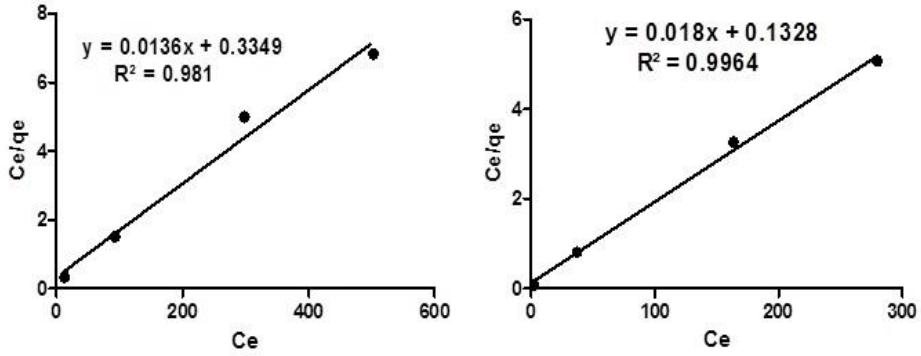

Fig. 3 Best fit plot between Ce and Ce/qe of a) F3 b) PB API

6.3 Pharmacoscintigraphy Evaluation

6.4.1 In-vitro release profile of radio labeled F3: The radiolabeled F3 was studied for *in-vitro* drug release in SGF for 2h and in SIF for 60 minutes to study the release pattern of drug. 1 ml samples were withdrawn at predetermined time intervals and measured for radioactive counts using gamma ray spectrometer (ECIL, India). The gamma counts were compared to drug release. The cumulative counts were plotted versus time (Fig. **4**).

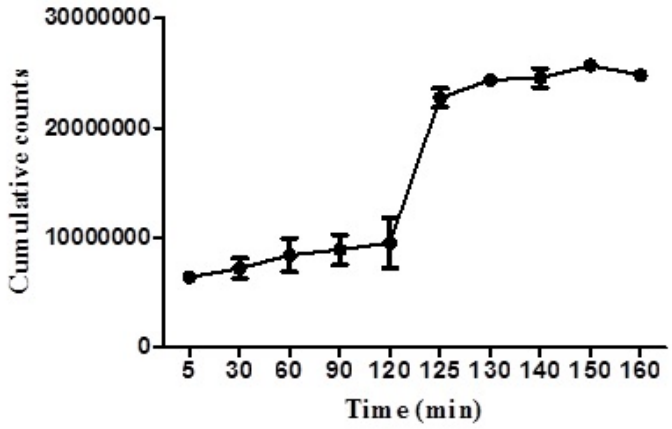

Fig. 4 *In-vitro* release profile of $^{99m}TcO_4^-$ loaded F3 (n=3)

6.4.2In-vivo pharamcoscintigraphy: Gamma scintigraphy was performed in rabbit model as discussed in 2.7.3. The static images of both control and test rabbit at predetermined time interval are shown in Fig. (**5**). Blood pool activity in both controls appears within 1 h and completely in 2h while in rabbit with test formulation, blood pool activity did not appear up to 1h and completely appeared in 3h. Analyzing dynamic images, a region of interest (ROI) was marked over chest cavity and counts/sec versus time graph was plotted (Fig. **6**) for examining blood pool activity .The images gave the clear pattern of disruption of control and test formulation with respect to time and GI transit of formulation.

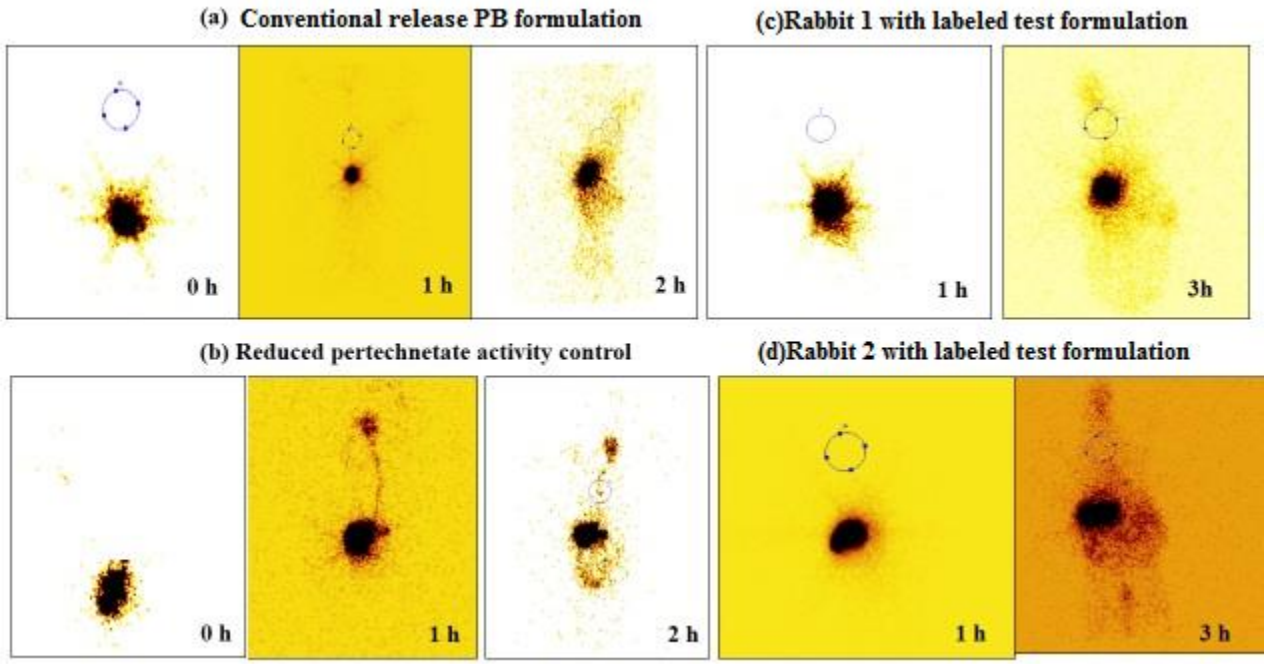

Fig. 5 Static images of rabbit after oral administration of (a) Labeled Conventional release PB formulation (b) Reduced pertechnetate activity capsule (c) Rabbit 1 with labeled Test formulation (d) Rabbit 2 with labeled Test formulation

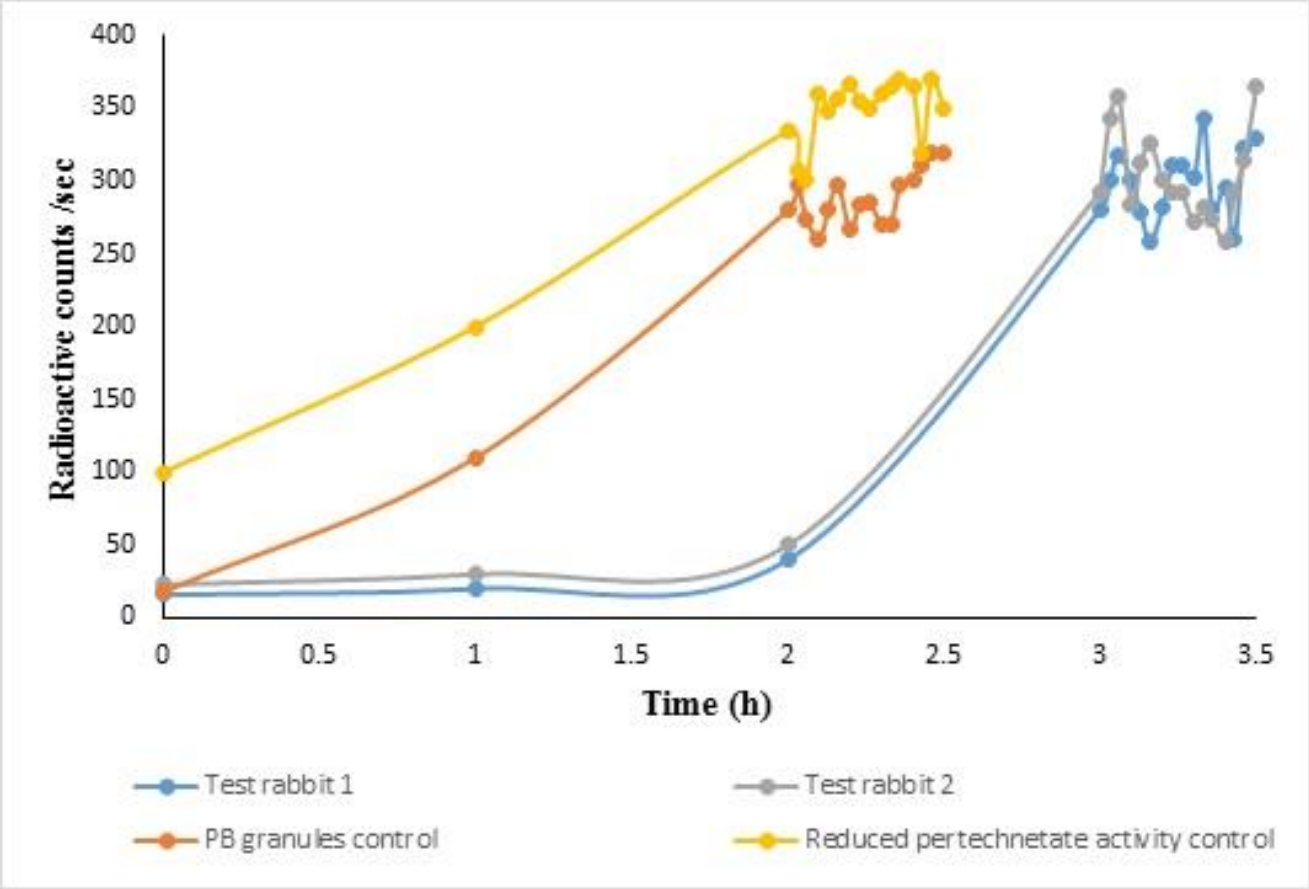

Fig. (6) Blood pool activity graph plotted between radioactive counts/sec and time

III. Development and characterization of orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides

A. Development and Validation of HPLC method of Ca-DTPA

1. Optimization of chromatographic conditions

Various trials to optimize chromatographic conditions were performed like variation in ratio of two mobile phases, flow rate etc. The results are mentioned in Table 1. A single HPLC isocratic gradient run in the proportion of 60:40 (TBA buffer: ACN), with flow rate of 1mLmin $^{-1,}$ injection volume 25 µL, column temperature 30ºC, wavelength maxima 280 nm operated for total chromatographic run time 10 min. was able to elute symmetrical peak shape of Ca-DTPA-Fe^{3+} complex with better resolution within optimal retention time of 5.6 min (Fig.7)

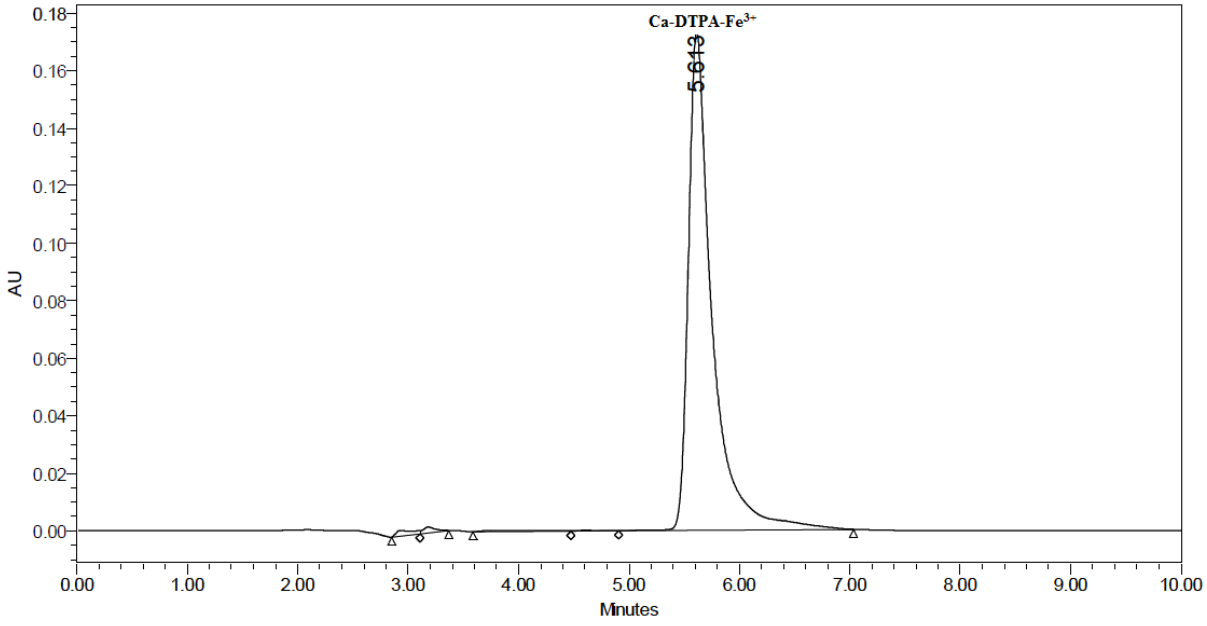

Fig. 7 Chromatogram of Ca-DTPA standard solution

2. Method validation

2.1 Specificity

After injection 10 µL of blank, standard and placebo solution in to HPLC system, the method was found to be very much selective for Ca-DTPA peak. No other interfering peak was found to be eluted at the retention time of drug peak (i.e. 5.6 min.) thus deducing the purity of analyte peak (Fig. 7-9).

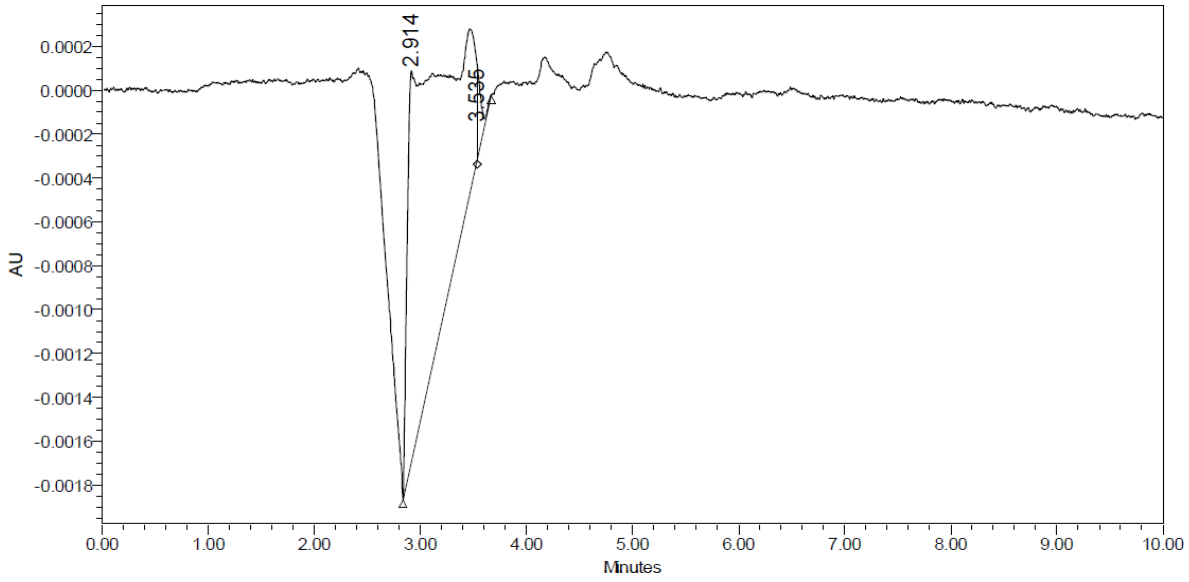

Fig. 8 Chromatogram of Blank solution

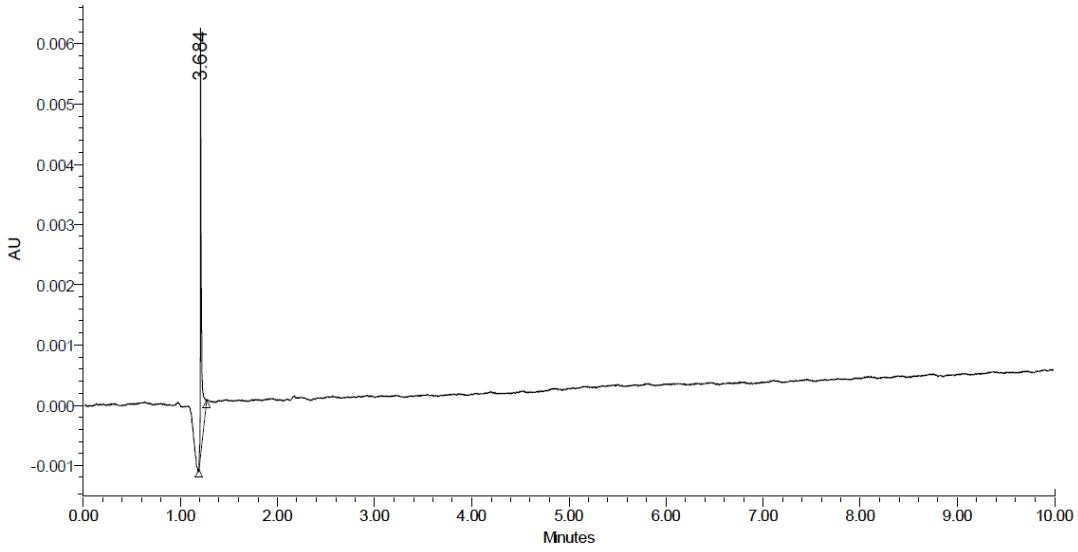

Fig. 9 Chromatogram of placebo solution

2.2 Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample

(Q2R1). The calibration curve was plotted between drug mean peak area under curve (AUC) and corresponding concentration of the drug. The linearity curve was plotted over range of concentration 2-100 µgmL^{-1} but linear regression coefficient close to 1 (i.e. 0.9997) was found over range of 2-20 µgmL^{-1} (Fig.4) for Ca-DTPA (Fig.10).

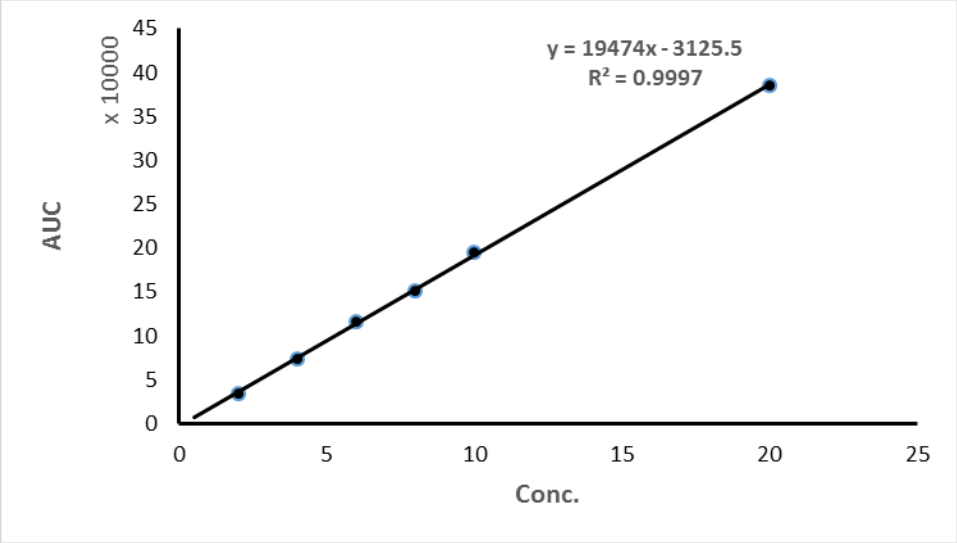

Fig.10 Calibration curve of Ca-DTPA using HLPC

2.3 Accuracy

The accuracy of developed method was estimated by spiking at three levels i.e. 50,100 and 150% to standard concentration of 4 µgmL^{-1}. The mean percent recovery at all three levels with three replicates at each level was found to be vary between (89.56-95.31%). The % RSD was found in the range of (0.3-0.8%). The results were found to comply with that of mentioned in Q2R1 guidelines. Thus the method can be used for routine analysis of drug.

2.4 Precision

The precision studies of the developed method was estimated by 1) repeatability assays of 3 standard concentrations /3 replicates (i.e.2, 6 and 10 µgmL^{-1}). The %RSD was found to be below 2% for all concentrations. 2) Intermediate precision was established by calculating %RSD of consecutive three days data for the same concentrations mentioned above and it was also found to be <2% . Both methods justified the precision of developed method.

2.5 LOD and LOQ

The limit of detection i.e. lowest detectable amount not necessarily quantitated while limit of quantitation i.e. lowest quantitative amount with approximate precision was developed as per method discussed in 2.5.5. The LOD was found to be 0.536 µgmL^{-1} and LOQ was found to be 1.62 µgmL^{-1}.

IV. In-vitro permeation study of Ca-DTPA

The membrane permeation studies were conducted using Franz diffusion apparatus with the help of Durapore PVDF membrane (pore size, 0.22 micrometer). The study was conducted for 4 h and percentage cumulative permeation of drug through barrier was calculated and plotted with respect to time (**Fig.11).**

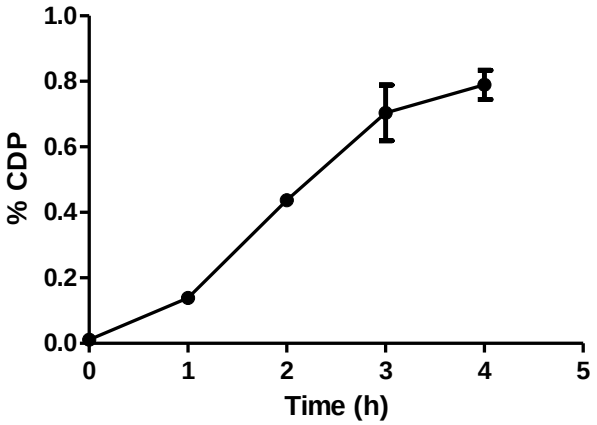

Fig. 11 Percent CDP v/s time plot of Ca-DTPA

V. Development and evaluation of CA-DTPA loaded SDEDDS

1. **Screening of excipients:** It was done based on solubility studies. Results are mentioned below in Table 1. On the basis of solubility studies: MCT was selected as oil component due to least solubility of drug in MCT so that maximum drug content remained in inner aqueous phase. Oleic acid was also added along with MCT as it was reports to enhance intestinal permeability. Span 80 (HLB 4.3) and Tween 80 (HLB 15) was chosen as lipophilic and hydrophilic emulsifiers on the basis of their HLB value in order to form stable SDEDDS system.

Vehicles	**Solubility of Ca-DTPA (mg /g)**

Castor oil	**0.03 ±0.00008**
Corn oil	**0.07 ±0.003**
Olive oil	**0.05 ±0.001**
MCT	**0.01 ±0.001**
Tween 80	**0.77 ±0.06**
Span 80	**0.68 0.001**

2. Formulation and development of Ca-DTPA loaded SDEDDS

Trial formulation was made with drug (100mg); inner aqueous phase (10%); Oil (70%) with MCT: Oleic acid (3:1); Tween 80 (10%)

3. **Parameters studied:**

3.1 **Visual observation**: Dull white; After 50 fold dilution with water within1 min: white (Fig.12).

Fig.12 Visual observation of SDEDDS system

3.2 **Optical Microscopy**: Morphology of SDEDDS formulation was observed after transformation in to w/o/w multiple emulsion under inverted fluorescence microscope (10x). Eosin was encapsulated to view drug loading inside inner core of multiple emulsion (Fig. 13).

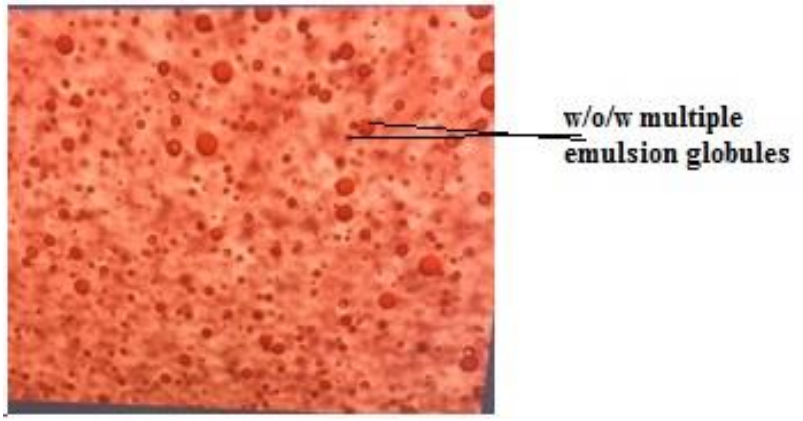

Fig. 13 Optical microscopy of SDEDDS after 100 fold dilution under inverted microscope at 10X (eosin dye encapsulated)

Discussion

Prussian blue is a well-known decorporation agent for removal of Cs-137 and ^{201}Tl. It is a crystal lattice that exchanges potassium for cesium and thallium at the surface of crystal [16]. Both ions follow enterohepatic circulation following the movement of potassium in the body i.e. first excreted into the intestine, reabsorbed from the gut into the blood and then to the bile where it is excreted again into the gut [17]. Thus the present study was aimed to formulate matrix tablet formulation of Prussian blue which can target thallium ions entering in to the gut through enterohepatic circulation and facilitates its excretion. Variants of granules (G1-G12) were formulated as mentioned and their optimization of granules were done by studying its release pattern in SGF and SIF. The optimized one showed no release of drug in SGF and complete empty of basket in SIF within 30-60 minutes. The *in-situ* physical crosslinking of Ca^{2+} with sodium alginate through ionotropic gelation attributed to prevent matrix disintegration in acidic media [18]. The optimized granules were compressed with variant concentration of PVPk30 as wet granulating agent in order to achieve desired hardness and optimum *in-vitro* release profile. The *in-vitro* release study of optimized one showed insignificant drug release at gastric pH and as formulation transcends to enteric pH, the erosion of entire formulation starts and complete release within 60 minutes. Calcium alginate matrix granules which were insensitive to acidic medium, get swollen and undergoe**s** erosion due to break down of gel structure at higher pH [19]. Thus, Alginate matrices showed pH dependent hydration, swelling and erosion behavior, thus provide pH dependent release formulation [18]. Further, the release data was fitted to various kinetic models and Hixson-crowell model was found to best fit our data with regression coefficient value very close to 1 i.e. 0.9996. This model gives us the pattern of drug release i.e. erosion dependent drug release where formulation was found to diminish proportionally such that the initial geometric form keeps constant all the time. Also, it can be assumed that the release of drug particles is by dissolution through polymeric matrix not by diffusion [20]. A study determined 24h equilibrium time attained by PB with thallium [21]. Thus 24h binding data was used to calculate the MBC from the slope of Langmuir isotherm using least squares linear regression (Fig. **4**). The primary mechanism of interaction for metal binding would be the ion exchange with the hydronium ions in the prussian blue crystal [21]. The maximum binding capacity of F3 and PB API to thallium was found to be 89.60 and 65.90 mg/g respectively. After applying student's t-test, the significant difference was found between maximum binding capacity of F3and PB API at

$p<0.05$. It was possibly due to the fact that the formulation contains other excipients like Calcium gluconate, sodium alginate which are contributing towards binding of metal ions and helps in decorporation. Further, the movement of F3 in the body and its release pattern was observed using pharmacoscintigraphy technique ($^{99m}TcO_4^-$ labeled). Technetium pertechnetate was used to radiolabel the formulation and reducing agent like stannous chloride was used to reduce pertechnetate ion where the oxidation state of technetium is reduced from 7 to presumably 4, the state in which technetium forms a complex with chelating molecule of interest [22]. Surface radiolabeling was done for labeling matrix granules wherein the matrix granule was completely wet, penetrated and labeled by reduced pertechnetate. This technique leads to heterogeneous radiolabeling which was better than homogenous one to some extent because in this technique maximum polymer comes in contact with activity. *In-vitro* release profile of radiolabeled F3 (Fig. **5**) showed biphasic release curve which is very much similar to *in-vitro* drug release profile of F3 (Fig. **2**) in terms of release pattern. The gamma counts released were compared to drug release from formulation. Approximately 25% of radioactivity was found to be released in SGF for 2h and complete release in SIF within 30-60 min. This pattern of radioactivity release also confirms efficient radiolabeling else complete activity might get released in SGF during 2h. This slow release of activity in SGF may be due to water soluble free pertechnetate leaching out from labeled formulation with no physical observation of PB release in SGF. Therefore, it can be predicted that PB matrix formulation did not release drug in stomach as radiolabeled formulation showed slow release of activity in SGF that too due to free pertechnetate. But in intestine the matrix formulation gets eroded and complete release of drug took place as gamma counts got increased in SIF. Thus, based on *in-vitro* analysis of radioactivity release pattern, *in-vivo* pharmacoscintigraphy was performed in rabbit where pH dependent release pattern of F3 was observed on the basis of blood pool activity of free pertechnetate with respect to time and position of formulation in the GIT. Gamma scintigraphy was performed and static images were acquired as mentioned in sec. 2.7.3. The control images clearly showed intact image of radiolabeled formulation at 0h while it starts disrupting within 1h and complete disruption within 2h. On the other hand test images of rabbit (n=2) showed intact radiolabeled G12 images up to 1h and 2h (not shown as not significant difference was found between 1& 2h) while complete blood pool activity was observed in 3h (Fig. **6**). Thus, the test granules remain intact in stomach and starts disruption in intestine only. The dynamic imaging of 15 frames each of 2 min was also done for test (at 3h) as well as control (at 2h) and ROI was marked over chest cavity for evaluating gamma counts over a

period of time during transit of test and control formulation in GIT. When, the gamma counts were plotted versus time gives the clear pattern of drug release *in-vivo* (Fig. **7**). Both control formulation showed steep increase in activity after 1h while test formulation showed after 2h only. The results of *in-vitro* and *in-vivo* release profiles of radiolabeled F3 were found to be superimposed over each other. At acidic pH (pH 1.2) *in-vitro* and in stomach free $^{99m}TcO_4^-$ leaches out at very slow rate while at basic pH (pH 6.8) *in-vitro* and in duodenum it comes out at faster rate suggesting erosion of matrix formulation completely. Therefore, it proves our concept that pH dependent matrix formulation was able to retain drug in stomach and deliver in intestine only at higher pH.

UV spectroscopic method of Ca DTPA was developed and validated using ion-pairing reaction with Ferric chloride. HPLC method was developed and validated for the quantification of drug in formulation *In-vitro* experimentation study was conducted with pure drug and found that the drug showed permeation only up to 0.6% when passed through membrane filter (pore size 0.22 micrometer).SDEDDS system for enhancing oral bioavailability of CA-DTPA is under formulation development and evaluation phase.

Conclusion

- pH dependent matrix release tablets of Prussian blue was successfully developed and characterized for their release in intestine.
- HPLC and UV spectroscopic method of Ca-DTPA was developed and validated as per ICH guidelines. Further *in-vitro* permeation study was conducted and the results showed up to 10% permeation. SDEDDS system for enhancing oral bioavailability of CA-DTPA is under formulation development trial phase

Future Plans

1. Stability studies of PB matrix formulation (on-going).

2. To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides (on-going).

3. To evaluate in-vivo efficacy of developed formulations.

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Research student	**Research Supervisor**	**Research Supervisor**
Vaishali Agrawal	**Dr. D.P. Pathak**	**Dr. A.K. Singh**
		Research Co-supervisor
		Dr. Nidhi Sandal

DATE: 24-09-20

PLACE: New Delhi

REPORT OF THE DOCTORAL STUDENT RESEARCH COMMITTEE (DSRC)

(Meeting held in hybrid mode on 07 Aug at 11:00 am)

Name of the Research Scholar: Ms. Vaishali Agrawal

Registration no.: 33/PhD/DIPSRU/2018

Conduct of the Research Scholar: The conduct of Ms. Vaishali Agrawal during the fourth DSRC period was found satisfactory.

Progress report: Attached

Overall Assessment of the progress and specific comments of the supervisor: *The progress of Ms. Vaishali Agrawal has been satisfactory*

Comments:

- The candidate has presented the detailed report of experimental methodology, results, discussion, summary and conclusion with respect to all the approved aim and objectives.
- The work has been completed in a very comprehensive manner with proper rationale.
- She has filed two patents from her research work with Indian Patent Office: one with **Application no.: 201913044367; dated 21 Nov 2019** and another one with **Application no.: 202311032095; dated 05 May 2023.** The candidate has published one research paper in SCIE journals of UGC list related to her PhD research work and another research paper currently under review. She has also presented two research papers in conferences.
- The suggestions given by experts during previous DSRC meetings have been properly incorporated by the scholar in her research work and presented in the fourth DSRC meeting.
- The DSRC recommends Ms. Vaishali Agrawal to carry out the writing of thesis work as she has done sufficient work to get the hypothesized outcomes during her PhD project.

Vibhav Garg

Dr. Vibhav Garg
Expert

could not attend

Prof. S.P. Agrawal
Expert

D.P. Pathak

Prof. D.P. Pathak
(Supervisor)
Professor, DIPSAR

Dr. A.K. Singh
(Co-supervisor)
Senior VP, AFSTIA

Dr. Nidhi Sandal
(Co-supervisor)
Sc. F, INMAS

Executive Summary (4th DSRC)

Name of Research Scholar: Vaishali Agrawal

Name of Supervisors: Prof. D.P. Pathak, Dr. A.K. Singh and Dr. Nidhi Sandal

Registration No.: 33/PhD/DPSRU/2018

Date of DSRC: 07 August 2023

Title of Thesis: Formulation Development of Various Agents to Improve Its Efficiency as Internal Decorporation Agent

Abstract of the thesis:

Introduction:

Cesium (Cs) and thallium (Tl) are the most devastating radionuclide produced during fission. The research work focused on formulation development of various agents to improve its efficiency as internal decorporation agents. Prussian Blue (PB) is very well known USFDA approved decorporation agent for cesium and thallium ions, available as "Radiogardase" conventional release dosage form. Thus oral pH dependent matrix formulation of PB based on in-situ gelation of sodium alginate coupled with calcium salts was developed and studied for its *in vitro* and *in vivo* efficacy with the objective to increase efficacy, target release, reduce dose and adverse effects. Another very well-known USFDA approved decorporation agent for actinides i.e. **Pentetate Calcium Trisodium (Ca-DTPA),** with low oral bioavailability (<3%) available in injection form only. Thus oral Ca-DTPA loaded SDEDDS system was designed with the objective to enhance its oral bioavailability.

Objectives:

1. To develop and characterize a PB dosage form which should release the drug in duodenum for decorporation.
2. To develop orally effective formulations of Ca-DTPA for decorporation of radioactive nuclides.
3. To evaluate *in vitro*/ *in vivo* efficacy of developed formulation.

Methodology:

Objective 1:

1. Development and Validation of UV spectrophotometric method of Prussian blue
2. Matrix Formulation development and *in vitro* characterization of developed formulation

- Granulation and Pre-compressional evaluation of granules
- Compression of optimized granules to matrix tablet (F1-F3)
- In-process quality control parameters of Matrix tablet (F1-F3) i.e. Hardness, Friability, Weight varia
  In vitro drug release study
- Kinetic Modelling, pH dependent Release study of optimized matrix tablet formulation in Citrate-Phosphate Buffer (pH range: 3-7)

Objective 2:

1. Development and validation of UV spectrophotometric and HPLC method of drug.
2. Solubility studies and Construction of pesudoternary phase diagrams
3. Preparation of oral SDEDDS dosage form of Ca-DTPA
4. *In vitro* characterization: Viscosity analysis, Assessment of self-double-emulsification performance of SDEDDS formulations, Emulsion droplet size analysis, *In vitro* permeation studies

Objective 3:

1. *In vitro* adsorption efficacy study by Atomic absorption spectroscopy
2. Pharmacoscintigraphy study of optimized PB matrix formulation
3. Stability studies
4. Toxicity studies and *In vivo* efficacy study of optimized PB matrix oral formulation
5. *In vivo* absorption and pharmacokinetics studies of Ca-DTPA loaded optimized (F4) SDEDDS formulation
6. Statistical Analysis

Results:

1. Oral pH dependent release matrix formulation of Prussian blue was successfully formulated with desired hardness and optimum *in vitro* release profile i.e. no drug release in acidic medium for 2h whereas complete empty of basket in basic medium within 30-60 minutes. Hixson-Crowell release model with r^2 (0.9906) confirmed the erosion based release mechanism. The maximum binding capacity (MBC) was found significantly higher (89.60mg Tl/g formulation) than that of PB API (65.90 mg Tl /g PB API). Pharmacoscintigraphy images confirmed intact formulation in stomach up to 2h and burst release in intestine thereafter. No significant toxicity was reported in formulation. *In vivo* efficacy study showed significant reduction of thallium in blood at 7th day, significant excretion of thallium level in feces up to 14 days and significantly less thallium content in urine samples at 7th day of formulation treatment Group with respect to control, API and Conventional release granules (CR) treatment group. By the end of 28 days, formulation treated rats showed significant reduction in Whole body retention to (13.79±.0.81%) when compared to positive control rats (30.34±0.63%), API treated rats (21.45±1.6%) and CR treated rats (19.23±0.22).
2. The optimized Ca-DTPA loaded SDEDDS formulation F4 (Aq. phase: 11.6%w,w; MCT & oleic acid: 70.9%w/w; Span 80:17.5%w/w; Lecithin:16%w/w and Tween 80 (10%w/w)) appeared bright yellow liquid which upon dilution appeared milky white within 2 min, particle size (501.7 nm), pdi value (0.044), zeta potential (-52 mV), entrapment efficiency (79.6±1.63), viscosity (72.2±1.8 mpA.s), significant high cumulative drug permeation (CDP) and 2.17 fold increase in apparent permeability coefficient. Pharmacokinetic studies in rats showed 1.17 fold increases in AUC of F4 and comparatively

higher plasma levels (Cmax) compared with pure drug administered orally. The Absolute (OF4, OD) and Relative bioavailability was found to be 14.52%, 12.35 % and 117.47% respectively.

Conclusion:

- The results exemplify oral pH dependent PB matrix formulation which achieved desirable target release at duodenum as calcium alginate well behaved as erodible matrix, able to retain drug at acidic pH and complete release at intestinal pH. *In vitro* binding and *In vivo* efficacy studies showed significant reduction in thallium levels of plasma, urine, whole body retention and major organs with increased fecal excretion of formulation treated groups. Thus we can conclude Prussian blue as a major antidote for thallium poisoning with better improvement in its efficacy with Prussian blue matrix oral formulation.
- Ca-DTPA-SDEDDS system was successfully explored for increasing oral bioavailability of Ca-DTPA. Thus improvising patient compliance and addressing mass casualties. The *in vitro* permeation study demonstrated a significant enhancement in drug permeation compared to the oral administration of the pure drug. *In vivo* studies showed that the SDEDDS system readily formed w/o/w multiple emulsion when diluted under mild agitation conditions, indicating its ability to transform into a suitable form for enhanced drug delivery in the gastrointestinal environment.

Outcomes of the overall work done:

Formulation development of USFDA approved agents for decorporation i.e. Prussian blue and Ca-DTPA was successfully done and evaluated for their *in vitro* and *in vivo* efficacy using various parameters.

Recommendations of 3rd DSRC	**Compliance of 3st DSRC (write clearly what changes were done)**
The presentation of the candidate was found satisfactory	Complied

Recommendations of UARC after 3rd DSRC	**Compliance**
The 3rd DSRC report was considered and Approved.	Complied

Recommendation of 4th DSRC:

- The candidate has presented the detailed report of experimental methodology, results, discuss summary and conclusion with respect to all the approved aim and objectives.
- The work has been completed in a very comprehensive manner with proper rationale.

- The candidate has published one research paper in SCIE journals of UGC list related to her PhD research work and another research paper currently under review.
- She has filed two patents from her research work with Indian Patent Office: one with **Application no.: 201913044367; dated 21 Nov 2019** and another one with **Application no.: 202311032095; dated 05 May 2023.**
- She has also presented two research papers in conferences.
- The suggestions given by experts during previous DSRC meetings have been properly incorporated by the scholar in her research work and presented in the fourth DSRC meeting.
- The DSRC recommends Ms. Vaishali Agrawal to carry out the writing of thesis work as she has done sufficient work to get the hypothesized outcomes during her PhD project.

Plagiarism report (Turnitin):

dsrc report

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List of Publications: Annexure

1. Agrawal V, Sandal N, Singh AK, Pathak DP. pH-dependent Release Matrix Oral Formulation of Prussian Blue to Improve its Efficacy as an Internal Decorporation Agent. Curr Drug Deliv. 2021;18(8):1162-1173. (I.F 3.7)
2. Agrawal V, Baghel R, Singh AK, Pathak DP, Sandal N. Development and Validation of LC–MS Method for the Estimation of N-Acetyl-Tryptophan and its Impurities Under Stress Conditions. Chromatographia. 2019 Oct 1;82(10):1467-77. ((I.F 2.21)
3. Agrawal V, Priyadarshani A, Pathak DP, Sandal N. Enhancing oral bioavailability of Ca-DTPA by Self Double Emulsifying Drug Delivery System (SDEDDS). Drug Development and Industrial Pharmacy. 2023 Dec 25:1-8.(I.F.3.19).

List of conference attended: Annexure

1.Poster presented on "pH Dependent Release Matrix Oral formulation of Prussian Blue to Improve its Efficacy as Internal Decorporation Agent." in 1st International conference on Innovations in Chemical. Biological and Pharmaceutical Sciences (ICBPS-2021) held at GLA University, Mathura on 26-27 Nov 2021.

2.Poster presentation on "Development and validation of LC-MS method for the estimation of N-Acetyl-Tryptophan and its impurities under stress conditions" in 70 th IPC held at Amity University, Noida on 21-23 Dec 20

Patents Filed: Annexure

1. A patent titled "pH Dependent Release Pharmaceutical Formulations and Process for its Preparation Thereof" has been filed with Indian Patent Office and accorded 201913044367 as application number dated 21 Nov.2019. Inventors are Vaishali Agrawal, Dr. D.P Pathak and Dr. Nidhi Sandal.
2. A patent titled "Oral Formulation of DTPA and its method of Preparation thereof" has been filed with Indian Patent Office and accorded 202311032095 as application number dated 05 May 2023. Inventors are Vaishali Agrawal, Dr. D.P Pathak and Dr. Nidhi Sandal.

Vaishali Agrawal

(Research scholar)

Prof. D.P Pathak

(Supervisor)